Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 21 July 2010, Revised 1 September 2009, Accepted 5 October 2009 Published online 9 November 2009 in Wiley Interscience

(www.interscience.wiley.com) DOI: 10.1002/jlcr.1694

Synthesis of labeled ambroxol and its major metabolites‡

Bachir Latli,[a]* Matt Hrapchak,[a] Heinz-Karl Switek,[b] Daniel M. Retz,[c] Dhileepkumar Krishnamurthy,[a] and Chris H. Senanayake[a]

Ambroxol is a mucolytic agent used in the treatment of respiratory diseases. Herein, we report the synthesis of carbon-14-labeled ambroxol with the radioactive atom(s) either on the benzylic carbon or uniformly in the cyclohexyl ring with specific activities of 59 and 81 mCi/mmol, respectively. We also describe the preparation of deuterium-labeled ambroxol, its deuterium-labeled tetrahydroquinazoline metabolite (DHTQ), carbon-13-labeled 3,5-dibromoanthranilic acid metabolite, as well as an unlabeled *O*-glucuronide conjugate.

Keywords: ambroxol; carbon-14; carbon-13; deuterium; metabolites

Introduction

Ambroxol is a mucolytic agent, marketed since the late 1970s, for the treatment of acute and chronic airway disorders characterized by the production of excess and thick mucus. The pharmacological and clinical data of ambroxol have recently been reviewed.[1] In Europe, ambroxol is marketed for acute upper respiration diseases, for the treatment of chronic bronchitis, and for neonatal respiratory distress syndrome.[2] It has been proposed as an anti-oxidant and an anti-inflammatory drug useful in preventing and treating influenza,[3,4] acute uncomplicated sore throat,[5] and other symptoms associated with rhinovirus infections.[6] It is hypothesized that ambroxol has a protective effect on pulmonary function after cardiopulmonary bypass.[7] Since 2002, ambroxol has been marketed as a lozenge for sore throat due to its local anesthetic effects.

Azmbroxol is a pharmacologically active phase I metabolite of the drug bromhexine, marketed since 1965 for the treatment of mild respiratory diseases. Oral bromhexine is metabolized in mammals to several compounds, and ambroxol is the most potent among several metabolites that retain pharmacological activity. As shown in Scheme 1, ambroxol is produced from a hydroxylation on the cyclohexyl ring and an *N*-demethylation.[8–12]

Herein, we report the synthesis of two versions of carbon-14-labeled ambroxol; one with the radioactive carbon in the benzylic position and the other with the radioactive carbon(s) uniformly distributed in the cyclohexyl ring to follow the metabolism of this moiety in mammals. Owing to the presence of two bromine atoms in the molecule, the preparation of labeled ambroxol with stable isotopes requires introducing more than six atomic mass units (amu) to easily discern it from the unlabeled drug in analytical studies. Thus, deuterium-labeled ambroxol with M+11 amu was prepared. The metabolite 6,8-dibromo-3-(*trans*-4-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline (DHTQ) was prepared with M+13 amu starting from deuterium-labeled ambroxol and deuterium-labeled formaldehyde. The other major metabolite, 3,5-dibromoanthranilic acid (DBAA),[13] labeled with carbon-13 was prepared. The potential metabolite 3,5-dibromoanthranilamide labeled with carbon-13 was also prepared. The unlabeled *O*-glucuronide conjugate of ambroxol was synthesized to aid in the identification and analytical comparisons with metabolites generated *in vivo*.

Results and discussion

In the synthesis of carbon-14-labeled ambroxol with the radioactive carbon in the benzylic position, Scheme 2, anthranilic acid [carboxylic-^{14}C]-, which is easily accessible in two steps from 1-bromo-2-nitrobenzene by carboxylation with $^{14}CO_2$ followed by reduction of the nitro group,[11] was reduced to the benzyl alcohol derivative using LAH in THF in 91% yield. Bromination with bromine in acetic acid gave 2-amino-3,5-dibromobenzyl alcohol in 38% radiochemical yield after flash chromatography purification. Oxidation of the benzyl alcohol derivative to the benzaldehyde was accomplished using MnO_2 in toluene at 80°C in 85% yield. Condensation of this aldehyde with *trans*-4-aminocyclohexanol in ethanol followed by reduction of the imine with $NaBH_4$ gave crude ambroxol. Purification by flash chromatography followed by treatment with a solution of HCl in dioxane gave the desired material in 51%

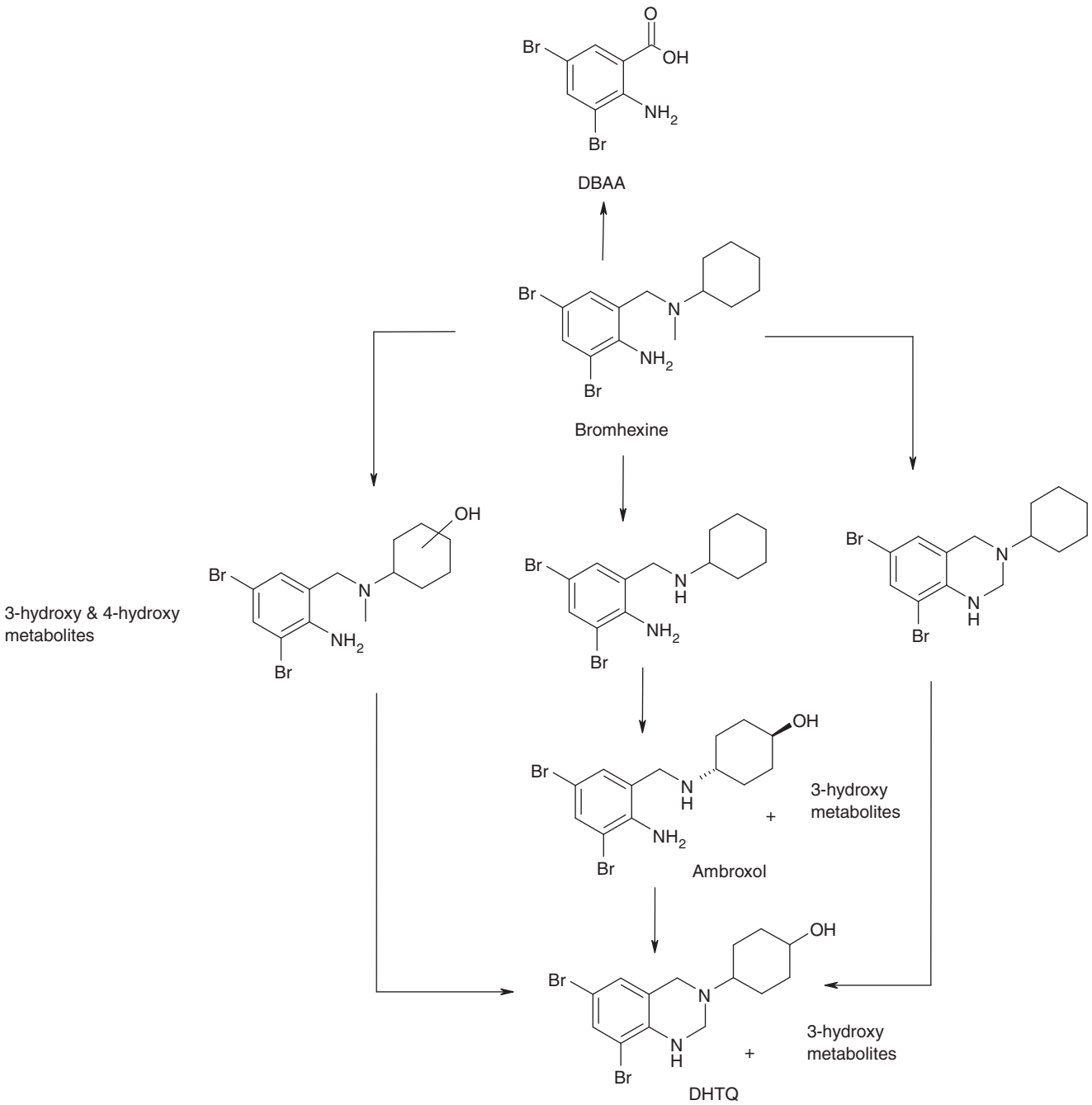
[a]*Department of Chemical Development, Boehringer Ingelheim Pharmaceuticals, Research and Development Center 900, Ridgebury Road, P.O. Box 368, Ridgefield, CT 06877-0368, USA*

[b]*Isotope Chemistry Group, Drug Metabolism and Pharmacokinetics, Boehringer Ingelheim Pharma GmbH & Co. KG, Birkendorfer Strasse 65, 88397 Biberach an der Riss, Germany*

[c]*Moravek Biochemicals, Inc., 577 Mercury Lane, Brea, CA 92621, USA*

**Correspondence to: Bachir Latli, Department of Chemical Development, Boehringer Ingelheim Pharmaceuticals, Research and Development Center, 900 Ridgebury Road, P.O. Box 368, Ridgefield, CT 06877-0368, USA. E-mail: Bachir.latli@boehringer-ingelheim.com*

‡*This paper is dedicated to Professor John E. Casida on his 80th birthday.*

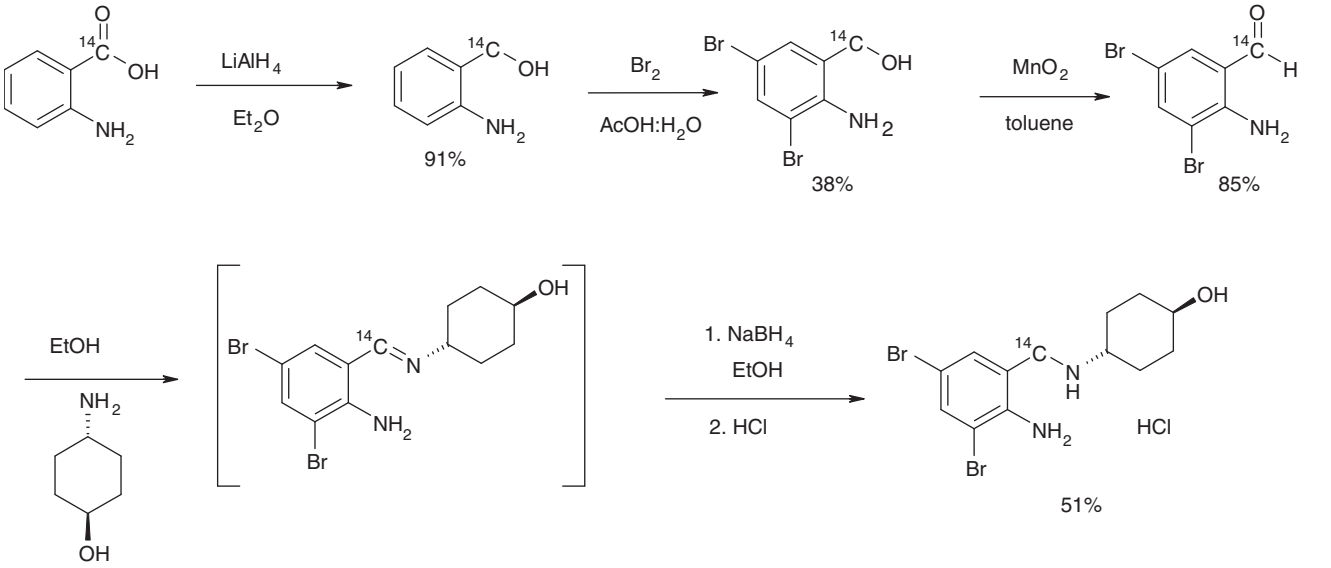


Scheme 1. Bromhexine and some of its metabolites as proposed in the literature.

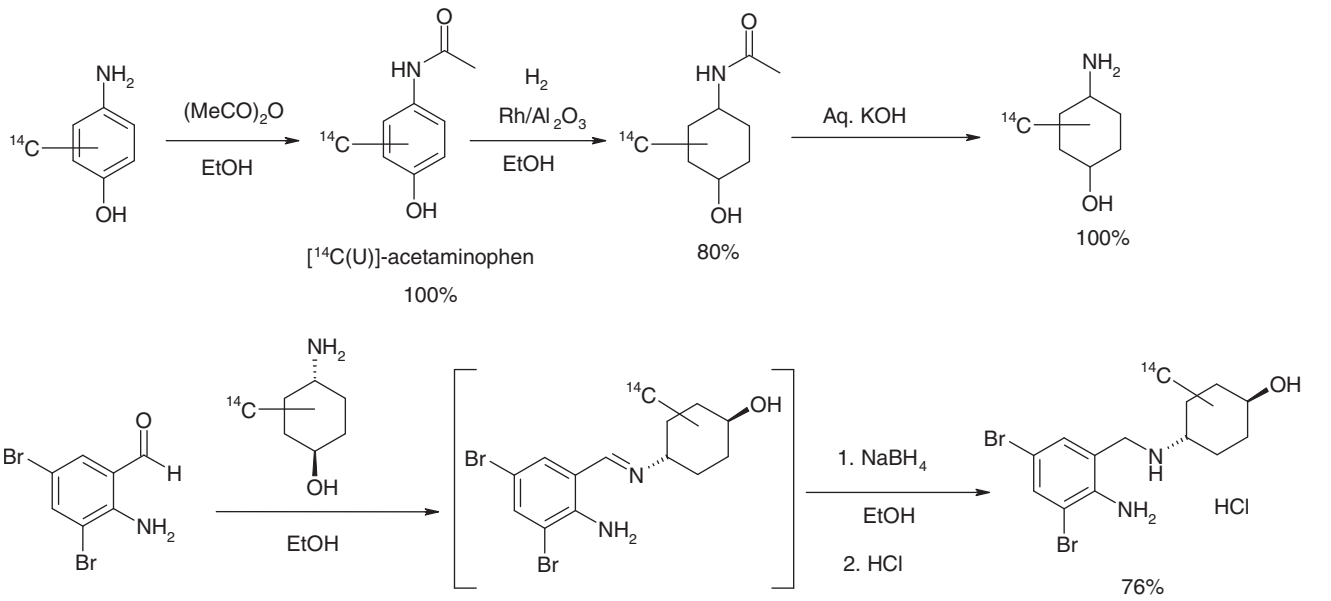
radiochemical yield and with a specific activity of 59.5 mCi/mmol and radiochemical purity of 98.3%.

In the second synthesis of carbon-14 ambroxol, Scheme 3, the radioactive atoms were incorporated in the cyclohexyl portion of the molecule so it can be easily followed during metabolism studies. Thus, uniformly labeled acetaminophen was reduced to *N*-acetyl-4-aminocyclohexanol under hydrogen gas in the presence of 5% Rh/alumina at 50 PSI.[14–16] The product was crystallized from acetonitrile to enrich the *trans*-isomer. Removal of the acetyl group was accomplished by heating at 110°C for 12 h with aqueous 10% KOH.[14] Condensation with 2-amino-3,5-dibromobenzaldehyde in ethanol followed by imine reduction in situ with $NaBH_4$, as previously described, gave carbon-14-labeled ambroxol contaminated with about 15% *cis*-isomer, which was easily removed by reverse phase HPLC. Labeled ambroxol was isolated with a specific activity of 81 mCi/mmol, but it was diluted to 49.4 mCi/mmol with commercial unlabeled ambroxol to minimize radiolysis.

To prepare deuterium-labeled ambroxol hydrochloride, Scheme 4, the commercially available [$^2H_7$]-4-aminophenol was first converted to [$^2H_4$]-acetaminophen using acetic anhydride and catalytic amounts of sodium dodecyl sulfate (SDS) in water.[17] Then, reduction under deuterium gas in the presence of rhodium on alumina at 60°C gave *N*-acetyl-4-amino-[$^2H_{10}$]-cyclohexanol in quantitative yield. At this stage, instead of performing a crystallization to remove the *cis*-isomer, the



Scheme 2. Synthesis of ambroxol, [benzyl, ^{14}C]-.

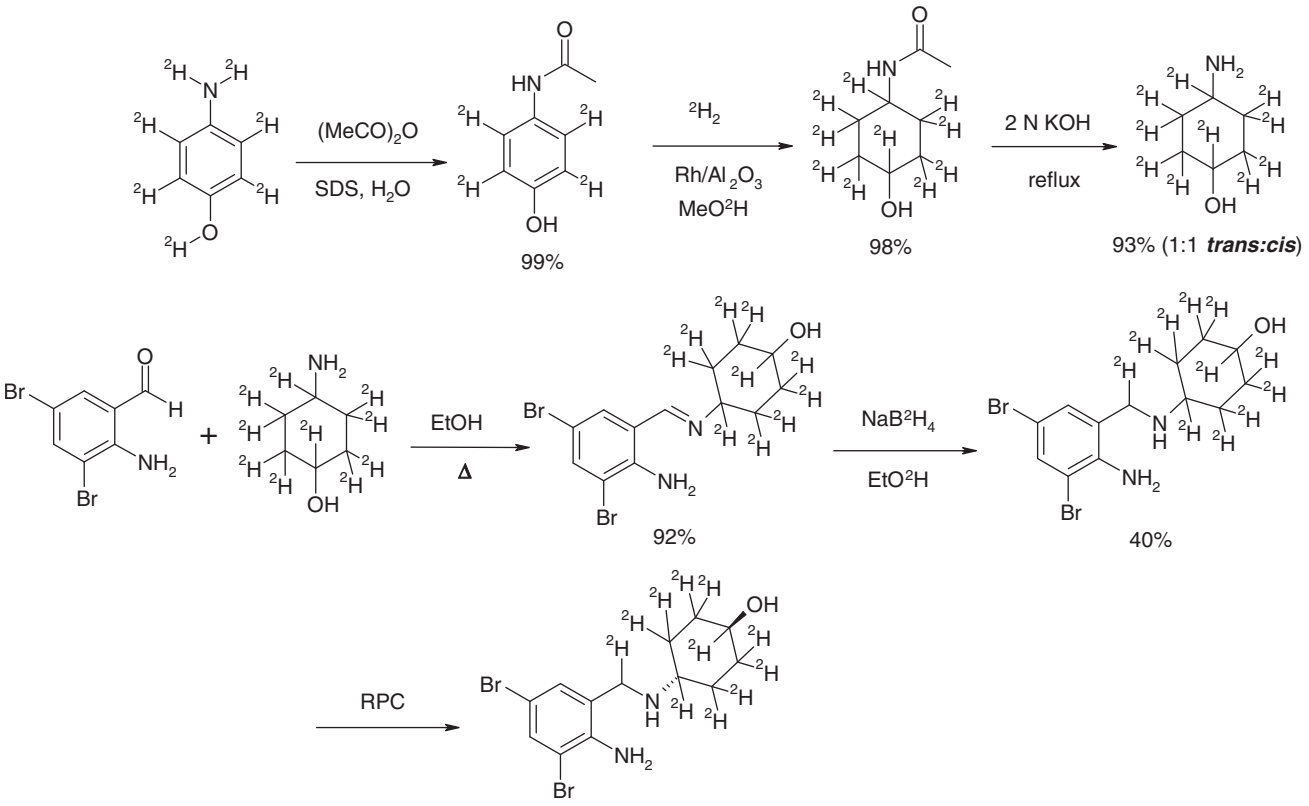


Scheme 3. Synthesis of ambroxol, [cyclohexyl, $^{14}C(U)$]-.

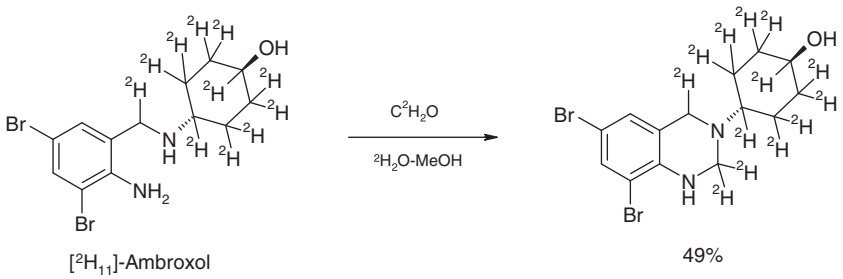
product was taken directly into the next step as a mixture of *cis*-and *trans*-isomers. Removal of the acetyl group was achieved using either aqueous 2N KOH in 93% yield as seen above, or 2.75N aqueous HCl in 89% yield. Coupling to 2-amino-3,5-dibromobenzaldehyde as seen above gave the labeled imine derivative in 92% yield. Reduction of the imine with sodium borodeuteride in ethanol gave [$^{2}H_{11}$]-ambroxol as a mixture of almost 1:1 *cis*: *trans* isomers. Isolation of the desired *trans*-isomer was accomplished by HPLC using a C18 reverse phase column and acetonitrile and water, which contained 0.1% perchloric acid and 0.1% phosphoric acid. The isotopic enrichment of the final product was more than 99%.

For the synthesis of the tetrahydroquinazoline metabolite (DHTQ), Scheme 5, [$^{2}H_{11}$]-ambroxol in methanol was heated to 80°C with a solution of [$^{2}H_{2}$]-formaldehyde in deuterated water to give after purification [$^{2}H_{13}$]-6,8-dibromo-3-(*trans*-4-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline ([$^{2}H_{13}$]-DHTQ) in 49% yield.[18]

To prepare labeled DBAA, the major metabolite of ambroxol, and the potential metabolite 3,5-dibromoanthranilamide, Scheme 6, [$^{13}C_{6}$]-aniline was first brominated to [$^{13}C_{6}$]-2,4-dibromoaniline using *N*-bromosuccinimide in methylene chloride in 76% yield. This compound was then iodinated with ICl in acetic acid at 60°C to 2,4-dibromo-6-iodo-[$^{13}C_{6}$]-aniline in 69% yield.[19] Cyanation with [^{13}C]-copper cyanide in pyridine at 100°C gave 2,4-dibromo-[$^{13}C_{7}$]-benzonitrile in 95% yield. Hydrolysis in either 70% aqueous sulfuric acid or in concentrated sulfuric acid gave 3,5-dibromo-[$^{13}C_{7}$]-anthranilic acid or



Scheme 4. Synthesis of [$^2H_{11}$]-ambroxol.



Scheme 5. Synthesis of deuterium-labeled tetrahydroquinazoline metabolite (DHTQ).

3,5-dibromo-[$^{13}C_7$]-anthranilamide in 53 and 47% yield, respectively. Alternatively, 2,4,6-tribromoaniline obtained from bromination of aniline in one step failed to give clean 3,5-dibromo-2-aminobenzonitrile using different cyanation conditions. When the amino group was transformed to a nitro group to help directing the ortho-cyanation, a mixture of products were obtained instead of the desired 2-nitro-3,5-dibromobenzonitrile.

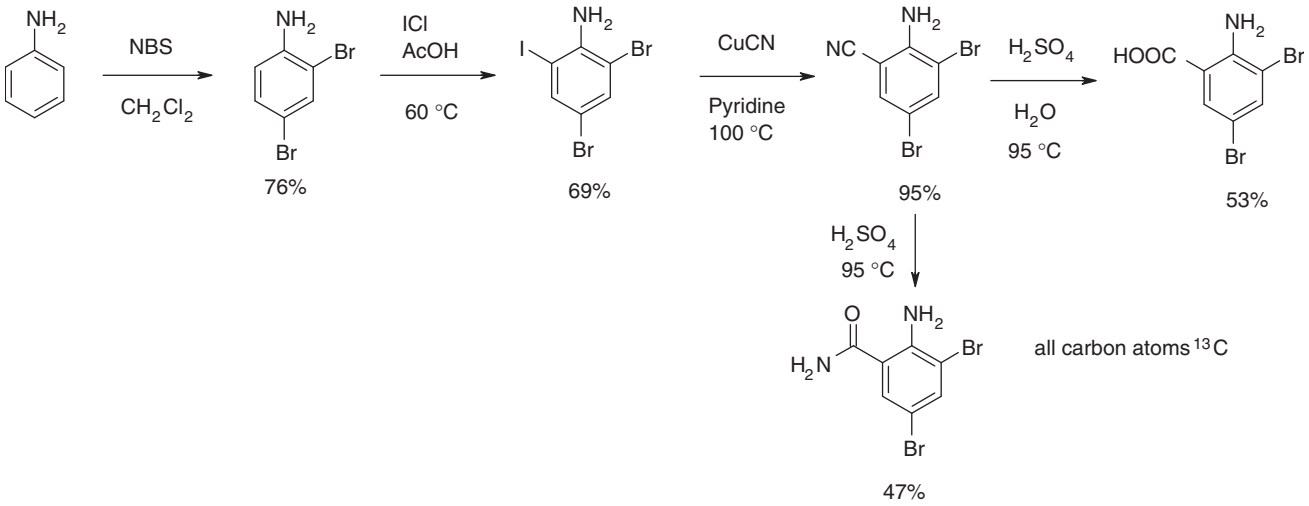
While the coupling constants between the aromatic protons of the unlabeled DBAA are about 2 Hz, large coupling constants are observed for the carbon-13-labeled DBAA between ^{13}C and ^1H. See Figure 1 below.

Lastly, to prepare the unlabeled *O*-glucuronide conjugate of ambroxol, Scheme 7, the free base of ambroxol was treated with acetobromo-D-glucuronic acid methyl ester in the presence of Ag_2CO_3 in toluene. Flash chromatography purification provided the desired product in a 30% yield. The structure was confirmed by 2D ROESY experiment. An NOE was observed as shown in the structure in Scheme 7. Ester hydrolysis with 1N aqueous NaOH in methanol gave mostly ambroxol after cleavage of the ether linkage to the sugar moiety. The desired product was obtained in low yield.

Experimental procedures

Materials and methods

Liquid scintillation counting was accomplished using a Beckman LS6500 and ready safe™ cocktail (Beckman, Fullerton, CA). The analytical HPLC radiopurity verification was carried out on a Supelco Discovery C18 (4.6 × 250 mm). UV detection was at 254 nm. The mobile phase consisted of A (water with 0.1% TFA), B (acetonitrile with 0.1% TFA), gradient: 0–5 min 0% B, 5–30 min 0–50% B, 30–30.1 min 50–100% B and hold to 40 min. For deuterium-labeled products, HPLC was carried out on an Eclipse



Scheme 6. Synthesis of 3,5-dibromo-[$^{13}C_7$]-anthranilic acid (DBAA) and the potential metabolite 3,5-dibromo-[$^{13}C_7$]-anthranilamide.

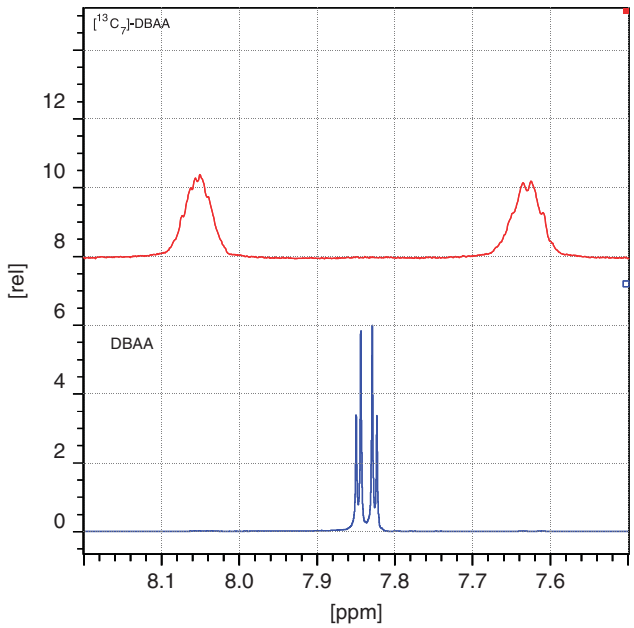


Figure 1. Proton NMR showing aromatic protons of [$^{13}C_7$]-DBAA and unlabeled DBAA in DMSO-d_6. Data were acquired using 500 MHz Bruker spectrometer.

XDB-C18 (4.6 × 150 mm). UV detection was at 220 nm. The mobile phase consisted of A (water with 0.1% H_3PO_4 and 0.1% $HClO_4$), B (acetonitrile with 0.1% H_3PO_4 and 0.1% $HClO_4$), gradient: 0–2 min 15–25% B, 2–3 min 25–30% B, 3–7.5 min 30–50% B. For carbon-13-labeled compounds HPLC was carried out on a Zorbax Eclipse XDB-C18 (4.6 × 150 mm). UV detection was at 254 nm. Mobile phase consisted of A (water with 0.1% TFA), B (acetonitrile with 0.1% TFA), gradient: 20–100% B over 20 min. For the glucuronide derivative of ambroxol, HPLC was carried out as described for carbon-13-labeled compounds with a gradient 5–100% B over 20 min. Mass spectra were acquired by a Hewlett-Packard auto sampler Series 1150, connected to a Micromass LCZ mass spectrometer in the ES mode. NMR spectra were recorded with a Bruker 400 MHz DPXB for the non-radioactive compounds and Bruker 300 MHz spectrometers for carbon-14-labeled compounds using deuterated methanol as a solvent and tetramethyl silane as the internal standard (Cambridge Isotope Laboratories, Andover, MA). Pre-coated TLC sheets (silica gel 60 F_{254}) were obtained from EM Science (Gibbstown, NJ). [$^{13}C_6$]-aniline, [$^2H_7$]-4-aminophenol, and [$^2H_2$]-formaldehyde ca. 20% wt% in ^2H_2O was purchased from Isotec (St. Louis, MO). All other reagents were purchased from Sigma-Aldrich.

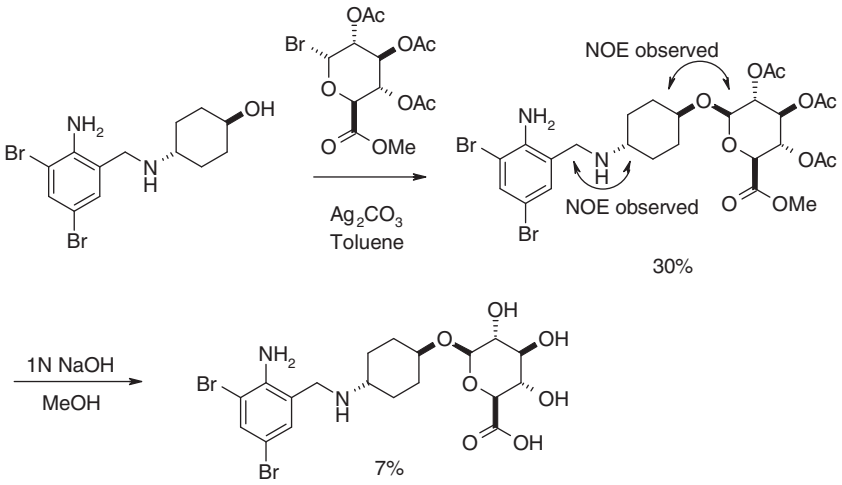
Synthesis

Synthesis of ambroxol, [benzyl, ^{14}C]-

2-Amino-[^{14}C]-benzyl alcohol: To a solution of anthranilic acid (265 mCi, 4.5 mmol) in anhydrous ether (30 mL) was added LAH (300 mg, 7.5 mmol) in ether (7.5 mL) with stirring in 20 min period. The mixture was refluxed for 1 h then cooled to room temperature. Excess LAH was destroyed by the addition of ethyl acetate, 2N aqueous NaOH and water (4 mL each). The organic phase was separated and the aqueous was extracted three times with ether. The combined extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give 263 mCi. Purification by silica gel chromatography using methylene chloride: methanol (15:1) gave 240 mCi of pure material in 91% radiochemical yield.

2-Amino-3,5-dibromo-[^{14}C]-benzyl alcohol: The above compound (240 mCi, 4.0 mmol) was dissolved in glacial acetic acid (6.2 mL) and water (2 mL). A solution of bromine (0.412 mL, 8.05 mmol) in glacial acetic acid (1.25 mL) was then added dropwise with stirring. A precipitate formed. Water (20 mL) was added and the mixture was stirred for 30 min, filtered, washed with water and dried under reduced pressure at 40°C. Flash chromatography purification using EtOAc:$CHCl_3$ as eluent (5–20%) gave 92 mCi of the desired product in 38% radiochemical yield.

2-Amino-3,5-dibromo-[^{14}C]-benzaldehyde: To the above compound (92 mCi, 1.56 mmol) in anhydrous toluene (26 mL) was added MnO_2 (0.88 g, 10.1 mmol) and stirred at 80°C for 1 h. TLC (2:1 $CHCl_3$: EtOAc) indicated no starting material. Filtration of the mixture gave a clear solution, which was concentrated *in vacuo* to give 78 mCi (85% yield) of material used directly in the following step.



Scheme 7. Synthesis of unlabeled ambroxol-*O*-glucuronide Conjugate.

Trans-4-(2-Amino-3,5-dibromo-[^{14}C]-benzylamino)-cyclohexanol: The above aldehyde (78 mCi, 1.32 mmol) and *trans*-4-aminocyclohexanol (173 mg, 1.5 mmol), which was obtained from the HCl salt by neutralizing and extracting with chloroform and isopropanol, in absolute ethanol (2.5 mL) was refluxed at 90°C over a period of 3 h. After cooling to room temperature, $NaBH_4$ (100 mg, 2.64 mmol) was added and stirred for 12 h. The mixture was evaporated to dryness and then diluted with aqueous 1N NaOH and extracted with CH_2Cl_2. The solvent was then removed *in vacuo* and the residue was purified by silica gel flash chromatography using 10% methanol in methylene chloride to give 44 mCi of ambroxol free base. The free base was dissolved in methanol and treated with a solution of 4N HCl in dioxane. The precipitate was concentrated and then crystallized from ethanol/ether to give 40 mCi of material with a specific activity of 59.5 mCi/mmol. ^{1}H NMR (500 MHz, CD_3OD)δ: 7.65 (d, $J = 2$ Hz, 1H), 7.42 (d, $J = 2$ Hz, 1H), 4.21 (s, 2H), 3.56 (m, 1H), 3.18 (m, 1H), 2.22 (m, 2H), 2.07 (m, 2H), 1.51 (m, 2H), 1.36 (m, 2H). HPLC, $R_t = 24.07$ min (98.3%), co-elutes with unlabeled commercial sample. MS, *m/z*: 378.8, 380.8, 382.8 (50%, 100%, 50% respectively); 379.8, 381.8, 383.8 (1:2:1).

Synthesis of ambroxol, [cyclohexyl-^{14}C(U)]-

N-Acetyl-4-aminophenol,[phenyl-^{14}C(U)]-: 4-Aminophenol [^{14}C(U)]-(428 mCi, 0.61 g, 5.59 mmol) in absolute ethanol (10 mL) was added acetic anhydride (0.6 g, 5.9 mmol) at room temperature. The solution was stirred for 15 min then concentrated *in vacuo* to give 0.859 g of a tan solid. Purification by silica gel flash chromatography using 10% methanol in methylene chloride gave 428 mCi of a white solid in 100% yield.

N-Acetyl-4-aminocyclohexanol,[cyclohexyl-^{14}C(U)]-: A mixture of the above compound (0.51 g, 3.33 mmol), 5% Rh on Al_2O_3 (0.13 g) in absolute ethanol (6.0 mL) was stirred under 50 psi of hydrogen for 12 h. The pressure dropped to 37 psi. TLC showed some starting material remaining. The hydrogenation bottle was pressurized again to 50 psi and stirred for 3 h. The reaction mixture was filtered and concentrated *in vacuo*. The residue was triturated with acetonitrile. Acetonitrile was then removed and the solid was crystallized from acetonitrile (2 mL). Removal of the solvent and drying the residue under reduced pressure gave 160 mg of *trans*-enriched isomer. The mother liquor gave after drying 267 mg of a mixture of *cis/trans* (3:2). Total of 427 mg or 80% yield.

Trans-4-Aminocyclohexanol,[cyclohexyl-^{14}C(U)]-: The *trans*-enriched material from above (0.16 g, 1.0 mmol) and potassium hydroxide (0.5 g, 8.9 mmol) in water (5 mL) was heated to 110°C for 12 h. After cooling to room temperature, the solution was saturated with NaCl and extracted four times with 4:1 chloroform/isopropanol. The combined extracts were dried over $MgSO_4$, filtered and concentrated to give 127 mg of material, which was used in the next step without further purification.

Trans-4-(2-Amino-3,5-dibromobenzylamino)-cyclohexanol,[cyclohexyl-^{14}C(U)]-: A mixture of the above crude material (0.127 g, 1.0 mmol), 2-amino-3,5-dibromobenzaldehyde (0.335 g, 1.2 mmol) in absolute ethanol (5 mL) was heated to 90°C for 5 h. After cooling to room temperature, $NaBH_4$ (0.09 g, 2.4 mmol) was added and the mixture was stirred at room temperature for 12 h. The product was isolated as mentioned above and purified by silica gel chromatography using chloroform to elute non-polar impurities then 5% MeOH/$CHCl_3$ to elute the desired product in 76% yield (289 mg) as syrup, or 62 mCi with a specific activity of 81 mCi/mmol. The product was dissolved in methanol and treated with 4N HCl in dioxane. After evaporation of the solvent, the residue was crystallized from ethanol/ether as seen above to give 35 mCi of material with a specific activity of 81 mCi/mmol. The specific activity was diluted to 49.4 mCi/mmol by adding 95 mg of ambroxol hydrochloride, purchased from Sigma-Aldrich, to 153 mg of labeled ambroxol in methanol and the solution was then concentrated and dried under vacuum. ^{1}H NMR (500 MHz, CD_3OD)δ: 7.65 (d, $J = 2$ Hz, 1H), 7.42 (d, $J = 2$ Hz, 1H), 4.21 (s, 2H), 3.56 (m, 1H), 3.18 (m, 1H), 2.22 (m, 2H), 2.07 (m, 2H), 1.51 (m, 2H), 1.36 (m, 2H). HPLC, 23.5 min (97.4%) co-elutes with an unlabeled commercial sample. MS: *m/z*: 378.9, 380.8, 382.2 (1:2:1), 384.8, 386.8, 388.8, 3918, 393.7, 395.8.

Synthesis of [$^2H_{11}$]-ambroxol

[$^2H_4$]-Acetaminophen: To a mixture of 4-aminophenol-[$^2H_7$] (5.0 g, 43.077 mmol) in water (180 mL) was added SDS (280 mg, 0.971 mmol) and the mixture was heated to 65°C until all the aminophenol was dissolved. To this dark solution acetic anhydride (6.5 mL, 67.4 mmol) was added and the mixture was stirred at this temperature for 30 min. Then, cooled to room temperature and stirred for 14 h. LC-MS showed no starting material. The dark mixture was extracted with ethyl acetate (3×200 mL), washed with brine, dried ($MgSO_4$), filtered and

concentrated *in vacuo* to give 6.78 g of purple solid. ^{1}HNMR (MeOH-d_4): 1.97(s, 3H). ^{13}C NMR (MeOH-d_4): 32.35, 115.84(t, J = 24.42 Hz), 122.97(t, J = 24.54 Hz), 131.45, 155.28, 171.37. LC-MS one single peak: MH^+ 156.13.

N-Acetyl-4-amino-[$^2H_{10}$]-cyclohexanol: A mixture of *[$^2H_4$]*-acetaminophen (6.25 g, 40.3 mmol) and Rh on alumina (10% Rh/Al_2O_3, 1.25 g) in deuterated methanol (36 mL) was stirred under a 25 PSI of deuterium gas (99.8 atom % ^{2}H) for 12 h. The mixture was filtered through a short pad of Celite, rinsed with methanol and concentrated *in vacuo* to give 6.98 g of a semi-solid. Purification by Combi-Flash Companion using 120 g column and 10–100% MeOH/CH_2Cl_2 gave 4.3 g of the desired product, LC-MS MH^+ = 168.21, and 2.3 g of material with traces of UV active contaminant. Total yield 98%.

4-Amino-[$^2H_{10}$]-cyclohexanol: A solution of the above material (1.0 g, 6.0 mmol) in aqueous KOH (10 mL, 2N) was heated to a gentle reflux for 12 h. The solution was cooled to room temperature and saturated with NaCl (3.5 g) and then extracted with $CHCl_3$: *i*PrOH (3:1), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give 0.7 g of a solid as a mixture of *cis*- and *trans*- isomers. ^{13}CNMR ($CDCl_3$) δ: 29.05 (m), 32.11 (m), 46.88 (m), 48.15 (m), 64.10 (m), 67.81 (m). LC-MS: ESI, MH^+ = 122.86.

Removal of the acetyl group was also performed using aq. 2.75N HCl. So a solution of the above material (1.27 g, 6.0 mmol) in aq. 2.75N HCl (15 mL) was heated to a gentle reflux for 12 h. The solution was cooled to room temperature and saturated with NaCl (3.5 g) and then extracted with $CHCl_3$: *i*PrOH (3:1), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give 0.85 g (89% yield) of material, which was used as it is in the next step.

2-Amino-3,5-dibromobenzylidene-4-amino-[$^2H_{10}$]-cyclohexanol: A mixture of *[$^2H_{10}$]*-4-aminocyclohexanol (0.7 g, 5.6 mmol) and 2-amino-3,5-dibromobenzaldehyde (1.4 g, 4.9 mmol) in absolute ethanol (10 mL) was heated to 80°C and stirred for 3 h. LCMS showed one single product with MH^+ = 387.42. The reaction was cooled to room temperature and concentrated *in vacuo* to give 1.988 g of a yellow solid, which was used as it is in the next step.

[$^2H_{11}$]-Trans-4-(2-amino-3,5-dibromobenzylamino)-cyclohexanol ([$^2H_{11}$]-Ambroxol): To a solution of the above imine (1.988 g, 5.148 mmol) in ethanol (25 mL), was added sodium borodeuteride (0.5 g, 11.72 mmol) at room temperature. Excessive gas evolution was observed. The reaction was stirred overnight under nitrogen atmosphere. No starting material was observed by LC-MS. Concentrated HCl (1.2 mL) was added to decompose excess NaB^2H_4, followed by aqueous 2N NH_4OH (50 mL) and extracted with $CHCl_3$ (100 mL × 3). The combined extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give 1.96 g of a solid. Purification by Combi-Flash on 120 g silicagel column and using up to 20% MeOH/CH_2Cl_2 gave 0.79 g of desired product in 40% yield as colorless film. LC-MS: MH^+: 387–393, unlabeled ambroxol MH^+ 377–382. The product was purified by Combi-Flash using a 120 g Redi-Sep C-18 column, which was first preconditioned using about 100 mL of MeCN. The compound was dissolved in methanol and applied to a 5 g C18 cartridge and then eluted using up to 25% MeCN in water (0.1% H_3PO_4 and 0.1% $HClO_4$). Tubes containing the desired isomer as judged form HPLC were then combined. HPLC, R_t = 4.48 min, 99.16%. (*Cis*-isomer R_t = 4.88 min). ^{1}HNMR ($CDCl_3$) of the free base: δ: 3.76(bs, 1H), 5.34(bs, 2H), 7.08(d, J = 2.20 Hz, 1H), 7.45(d, J = 2.20 Hz, 1H). ^{13}CNMR ($CDCl_3$): 29.10(m), 31.77 (m), 49.14(t, J = 20.41 Hz, benzylic CHD), 53.50(m), 68.62(m), 107.25, 109.37, 125.80, 130.24, 131.80, 142.85. MS-HR: MH^+ calculated 388.05473, found 388.05491. Traces of [$^2H_{10}$]- and [$^2H_9$]-ambroxol were observed.

Synthesis of [$^2H_{13}$]-6,8-Dibromo-(3-trans-4-hydroxycyclohexyl)-1,2,3,4-tetra-hydroquinazoline ([$^2H_{13}$]-DHTQ)

A solution of *[$^2H_{11}$]*-ambroxol (160 mg, 0.376 mmol) in methanol (2 mL) was treated with a solution of formaldehyde-$^2H_2$, 98 atom% ^{2}H, ca. 20 wt.% in ^2H_2O (2.0 g, 12.5 mmol). The resultant was heated to 80°C for 2 h. LC-MS showed the presence of new product MH^+: 401–407. After cooling to room temperature, the solution was concentrated *in vacuo* to give 221 mg of yellowish viscous oil. The crude was dissolved in $CHCl_3$ (10 mL) and treated with a solution of 2N NH_4OH (10 mL), extracted with $CHCl_3$, dried over $MgSO_4$, filtered and concentrated *in vacuo* to give 180 mg of material. Purification by flash chromatography using 12 g disposable silica gel column and 1–5% MeOH/CH_2Cl_2 gave 75 mg of pure product as a white solid in 49% yield. ^{1}H NMR ($CDCl_3$) δ: 3.90 (bs, 1H), 4.37(bs, 1H), 7.38(d, J = 2.10 Hz, 1H), 6.96(d, J = 2.10 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ: 27.44(m), 32.88(m), 49.65(t, J = 20.63 Hz), 51.86(m), 71.88(m), 79.62(m), 108.15, 109.08, 122.97, 128.89, 132.29, 140.00. LCMS: *m/z* : 401 to 408.

Synthesis of 3,5-dibromo-[$^{13}C_7$]-anthranilic acid ([$^{13}C_7$]-DBAA) and 3,5-3,5-dibromo-[$^{13}C_7$]-anthranilamide

2,4-dibromo-[$^{13}C_6$]-aniline: NBS (7.2 g, 43 mmol) was added to a solution of [$^{13}C_6$]-aniline (2.0 g, 21.5 mmol) CH_2Cl_2 (30 mL) at 0°C. The mixture was warmed to room temperature over 15 min, then stirred overnight. The reaction was quenched by the addition of H_2O, extracted with CH_2Cl_2 (10 mL × 3), dried over Na_2SO_4 and concentrated giving a light brown solid. The solid was diluted with EtOH (8 mL), warmed to dissolve the solid, then H_2O (7 mL) was added and the mixture was allowed to cool to room temperature. The solid was collected by vacuum filtration to give 2,4-dibromo-[$^{13}C_6$]-aniline (4.1 g, 76%) as light brown needles. HPLC: 11.1 min. LCMS: *m/z* 256. ^{1}H NMR (400 MHz, $CDCl_3$)δ: 7.53 (dm, J = 170 Hz, 1H), 7.19 (dm, J = 166 Hz, 1H), 6.64 (m, 1H), 4.59 (bs, 2H). ^{13}C NMR (100 MHz, $CDCl_3$)δ: 143 (t, J = 64 Hz), 134 (t, J = 66 Hz), 131 (t, J = 60 Hz), 117 (t, J = 60 Hz), 110 (t, J = 64 Hz).

2,4-dibromo-6-iodo-[$^{13}C_6$]-aniline: ICl (0.946 g, 5.84 mmol) in acetic acid (2 mL) was added to a solution of 2,4-dibromo-[$^{13}C_6$]-aniline (1.5 g, 5.84 mmol) in acetic acid (10 mL). The mixture was heated at 60°C for 5 min. Then H_2O (5 mL) was added and the mixture was heated to 90°C for 1 h. The mixture was cooled to room temperature and the solid was collected by vacuum filtration to give 2,4-dibromo-6-iodo-[$^{13}C_6$]-aniline (1.56 g, 69%) as a light brown solid. HPLC: 15.5 min. ^{1}H NMR (400 MHz, $CDCl_3$)δ: 7.69 (dm, J = 172 Hz, 1H), 7.53 (dm, J = 172 Hz, 1H), 4.59 (bs, 2H). ^{13}C NMR (100 MHz, $CDCl_3$)δ: 143 (t, J = 68 Hz), 140 (t, J = 64 Hz), 135 (t, J = 68 Hz), 109 (t, J = 64 Hz), 107 (t, J = 68 Hz), 83 (dd, J = 64, 68).

2-Amino-3,5-dibromo-[$^{13}C_7$]-benzonitrile: A mixture of 2-iodo-4,6-dibromo-[$^{13}C_6$]-aniline (0.75 g, 1.96 mmol) and CuCN (99 atom % ^{13}C, 0.355 g, 3.92 mmol) in pyridine (15 mL) was heated in a sealed vial at 100°C for 14 h. The mixture was cooled to room temperature and diluted with EtOAc, washed sequentially with H_2O, brine, dried over Na_2SO_4 and concentrated to give a brown solid. The solid was diluted with 25% EtOAc/Hexane (50 mL) and stirred overnight. The filtrate was then concentrated to give 2-amino-3,5-dibromo-[$^{13}C_7$]-benzonitrile (0.53 g, 95%) as an off white solid. HPLC: 11.2 min. ^{1}H NMR (400 MHz, $CDCl_3$)δ: 7.73 (dm, J = 171 Hz, 1H), 7.48 (dm, J = 169 Hz, 1H), 4.90 (bs, 2H). ^{13}C NMR (100 MHz, $CDCl_3$)δ: 144 (t, J = 68 Hz), 137 (t, J = 64 Hz),

131 (dd, $J = 64$, 66 Hz), 113 (d, $J = 82$ Hz), 107 (t, $J = 68$ Hz), 106 (t, $J = 64$ Hz), 96 (m).

2-Amino-3,5-dibromo-[$^{13}C_7$]-anthranilic acid ([$^{13}C_7$]-DBAA): Concentrated H_2SO_4 (5 mL) and H_2O (2 mL) was added to 2-amino-3,5-dibromo-[$^{13}C_7$]-benzonitrile (117 mg, 0.41 mmol), the mixture was heated at 95°C overnight. The mixture was cooled to room temperature, poured over ice and the pH was adjusted to 4 with 3N NaOH. The mixture was extracted with EtOAc and the combined organic extracts were washed with brine, dried over Na_2SO_4 and concentrated to give a light brown solid. The solid was recrystallized from EtOH/H_2O to give 2-amino-3,5-dibromo-[$^{13}C_7$]-benzoic acid (66 mg, 53%) as a light brown solid. HPLC: 10.4 min. LCMS: *m/z* 301. ^{1}H NMR (400 MHz, d_6-DMSO)δ: 13.37 (bs, 1H), 7.84 (dm, $J = 169$ Hz, 1H), 7.81 (dm, $J = 169$ Hz, 1H), 6.88 (bs, 2H). ^{13}C NMR (100 MHz, d_6-DMSO)δ: 168 (d, $J = 72$ Hz), 147 (t, $J = 65$ Hz), 138 (t, $J = 65$ Hz), 133 (t, $J = 65$ Hz), 112 (m), 110 (t, $J = 65$ Hz), 105 (t, $J = 65$ Hz).

3,5-Dibromo-[$^{13}C_7$]-anthranilamide: Concentrated H_2SO_4 (10 mL) was added to 2-amino-3,5-dibromo-[$^{13}C_7$]-benzonitrile (405 mg, 1.43 mmol) and the mixture was heated at 95°C for 14 h. After cooling to room temperature, the mixture was poured into ice and the pH was adjusted to 4 with 3N NaOH. The mixture was extracted with EtOAc and the combined extracts were washed with brine, dried over Na_2SO_4 and concentrated to give a light brown solid. The solid was purified by silica gel flash chromatography (20–100% EtOAc/Hexane) followed by crystallization from EtOH/H_2O to give 2-amino-3,5-dibromo-[$^{13}C_7$]-benzoic acid amide (203 mg, 47%) as a light brown solid. HPLC: 8.2 min. LCMS *m/z:* 300. ^{1}H NMR (400 MHz, d_6-DMSO)δ: 8.03 (bs, 1H), 7.78 (dm, $J = 82$ Hz, 1H), 7.73 (dm, $J = 82$ Hz, 1H), 7.45 (bs, 1H), 6.78 (bs, 2H). ^{13}C NMR (100 MHz, d_6-DMSO)δ: 169 (d, $J = 63$ Hz), 146 (t, $J = 64$ Hz), 137 (t, $J = 64$ Hz), 131 (t, $J = 63$ Hz), 117 (q, $J = 64$ Hz), 110 (t, $J = 69$ Hz), 105 (t, $J = 64$ Hz).

Synthesis of unlabeled O-glucuronide conjugate of ambroxol

(2R,3R,4S,5S,6S)-2-((1R,4R)-4-(2-Amino-3,5-dibromobenzylamino)-cyclohexyloxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate: A mixture of ambroxol (250 mg, 0.66 mmol), aceto-bromo-D-glucuronic acid methyl ester (262 mg, 0.66 mmol), and Ag_2CO_3 (182 mg, 0.66 mmol) in toluene (5 mL) was stirred at room temperature overnight. HPLC showed only recovered starting material. The mixture was heated at 100°C overnight. The mixture was cooled to room temperature, filtered (Celite) and concentrated to give yellow oil. The crude residue was purified using CombiFlash Companion (40 g disposable silica gel column and 0–10% MeOH/CH_2Cl_2) to give a yellow oil, which was further purified using CombiFlash Companion (12 g disposable column, 20–60% EtOAc/CH_2Cl_2) to give (2R,3R,4S,5S,6S)-2-((1R,4R)-4-(2-amino-3,5-dibromobenzylamino)-cyclohexyloxy)-6-(methoxycarbonyl)-tetrahydro-2H-pyran-3,4,5-triyl triacetate (140 mg, 30%) as a light yellow solid. HPLC: 13.3 min. LCMS: *m/z* 694. ^{1}H NMR (400 MHz, $CDCl_3$)δ: 7.46 (d, $J = 2.0$ Hz, 1H), 7.08 (d, $J = 2.0$ Hz, 1H), 5.31 (bs, 2H), 5.23 (m, 2H), 4.96 (dd, $J = 7.5$, 9 Hz, 1H), 4.64 (d, $J = 7.5$ Hz, 1H), 4.02 (d, $J = 9.5$ Hz, 1H), 3.76 (m, 2H), 3.75 (s, 3H), 3.62 (m, 1H), 2.46 (m, 1H), 2.03 (s, 3H), 2.01 (s, 6H), 1.95 (m, 4H), 1.42 (m, 2H), 1.12 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$)δ: 170.7, 169.8, 169.6, 167.8, 144.3, 133.7, 131.8, 127.2, 110.9, 108.8, 99.9, 78.5, 77.7, 73.1, 72.6, 71.9, 70.0, 55.6, 53.3, 51.1, 31.7, 31.0, 30.3, 21.1, 21.0.

(2S,3S,4S,5R,6R)-6-((1R,4R)-4-(2-Amino-3,5-dibromobenzylamino)-cyclohexyloxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid: 1N NaOH (1 mL) was added to a solution of the above material (110 mg, 0.16 mmol) in MeOH (5 mL) at room temperature. The mixture was allowed to stir at room temperature for 14 h, and then concentrated to give dark oil. The crude residue was purified by silica gel chromatography (5–15% MeOH/CH_2Cl_2), and then further purified by preparative HPLC (Agilent, 10–95% MeCN/H_2O (0.1%TFA)) to give a light yellow oil. LCMS revealed the product was the methyl ester, *m/z* 568. This oil was then diluted with MeOH (1 mL), THF (1 mL) and 1N NaOH (1 mL) was added at room temperature. The mixture was allowed to stir at room temperature for another 14 h to give the desired product and ambroxol. The mixture was concentrated then diluted with water, extracted with Et_2O to remove ambroxol. The aqueous layer, which contained the desired product, was then lyophilized to give a white solid. The solid was diluted with water, and the pH was adjusted to neutral with 3N HCl solution. The solution was then passed through a pre-washed SPE (C18) cartridge and the product was eluted using MeOH. Concentration in vacuo gave (2S,3S,4S,5R,6R)-6-((1R,4R)-4-(2-amino-3,5-dibromobenzylamino)-cyclo-hexyloxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (6 mg, 7%) as an off white solid. HPLC: 9.2 min. LCMS: *m/z* 554. ^{1}H NMR (400 MHz, CD_3OD)δ: 7.38 (d, $J = 2.0$ Hz, 1H), 7.15 (d, $J = 2.0$ Hz, 1H), 4.46 (bs, 2H), 4.26 (d, $J = 8.0$ Hz, 1H), 3.79 (m, 2H), 3.66 (m, 1H), 3.42 (d, $J = 9.0$ Hz, 1H), 3.28 (m, 2H), 3.05 (dd, $J = 8.0$, 9.0 Hz, 1H), 2.56 (m, 1H), 1.99 (m, 4H), 1.27 (m, 2H), 0.77 (m, 2H).

Conclusion

We have prepared carbon-14-labeled ambroxol with the radioactive carbon in either the benzylic position or uniformly distributed in the cylcohexyl ring with specific activities of 59.5 and 81 mCi/mmol, respectively. Deuterium-labeled ambroxol with an increase of 11 amu and its metabolite the tetrahydroquinazoline (DHTQ) with 13 amu were also prepared. The major metabolite of ambroxol, DBAA as well as the potential metabolite 3,5-dibromoanthranilamide with all carbon C13 were also prepared. The synthesis of *O*-glucuronide conjugate of ambroxol was synthesized as well to be used in the identification and in analytical studies.

Acknowledgement

We are in debt to our colleagues Dr Tom MacGregor and Peter Grob for critical reading of this manuscript, to Scott Pennino for obtaining high-resolution mass spectra for compounds labeled with stable isotopes, and to Dr Nelu Grinberg and Dr Ma Shengli for HPLC analysis of ambroxol isomers, and Dr Nizar Haddad for technical assistance.

References

[1] M. Malerba, B. Ragnoli, *Expert Opin. Drug Metab. Toxicol.* **2008**, *4*, 1119–1129.
[2] K. Nobata, M. Fujimura, Y. Ishiura, S. Myou, S. Nakao, *Clin. Exp. Med.* **2006**, *6*, 79–83.
[3] B. F. Gibbs, W. Schmutzler, I. B. Vollrath, P. Brosthardt, U. Braam, H. H. Wolff, G. Zwadlo-Klarwasser, *Inflamm. Res.* **1999**, *48*, 86–93.
[4] W. Gaida, K. Klinder, K. Arndt, T. Weiser, *Neuropharmacology* **2005**, *49*, 1220–1227.
[5] C. de Mey, H. Peil, S. Kölsch, J. Bubeck, J.-M. Vix, *Arzneim-Forsch* (*Drug Res.*) **2008**, *58*, 557–568.
[6] E. Anke, P. Liane, WO 2007/023072 A2.

[7] M. M. Ulas, M. Hizarci, A. Kunt, K. Ergun, S. S. Kocabeyoglu, K. Korkmaz, G. Lafci, S. Gedik, K. Cagli, *J. Cardiovasc. Pharmacol.* **2008**, *52*, 518–523.
[8] L. A. Meijer, J. C. M. Verstegen, S. Bull, J. Fink-Gremmels, *J. Vet. Pharmacol. Ther.* **2004**, *27*, 219–225.
[9] E. Schraven, F. W. Koss, J. Keck, G. Beisenherz, *Eur. J. Pharmacol.* **1967**, *1*, 445–451.
[10] Z. Kopitar, R. Jauch, R. Hankwitz, H. Pelzer, *Eur. J. Pharmacol.* **1973**, *21*, 6–10.
[11] R. Jauch, R. Hankwitz, *Arzneim-Forsch. (Drug Res.)* **1975**, *25*, 1954–1958.
[12] J. A. A. Jonckheere, L. M. R. Thienpont, A. P. Deleenheer, P. De Backer, M. Debackere, F. M. Belpaire, *Biomed. Mass Spectrom.* **1980**, *7*, 582–587.
[13] N. Ishiguro, C. Senda, W. Kishimoto, K. Sakai, Y. Funae, T. Igarashi, *Xenobiotica* **2000**, *30*, 71–80.
[14] T. P. Johnston, G. S. McCaleb, J. A. Montgomery, *J. Med. Chem.* **1975**, *18*, 634–637.
[15] J. H. Billman, J. A. Buehler, *J. Am. Chem. Soc.* **1953**, *75*, 1345–1346.
[16] H. K. Hall Jr, *J. Am. Chem. Soc.* **1958**, *80*, 6412–6420.
[17] S. Naik, G. Bhattacharjya, B. Talukdar, B. K. Patel, *Eur. J. Org. Chem.* **2004**, 1254–1260.
[18] J. Keck, *Liebigs Ann. Chem.* **1967**, *707*, 107–111.
[19] P. D. Leeson, R. Baker, R. W. Carling, N. R. Curtis, K. W. Moore, B. J. Williams, A. C. Foster, A. E. Donald, J. A. Kemp, G. R. Marshall, *J. Med. Chem.* **1991**, *34*, 1243–1252.