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10th International Symposium on the Synthesis and Applications of Isotopes and Isotopically Labelled Compounds—The Role of Isotopes in Pharmacokinetics and Drug Metabolism

Session 15, Wednesday, June 17, 2009

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Abstract: Conducting ADME studies on new drug candidates remains one of the primary uses of isotopically labelled compounds. In this session, the synthesis of isotopically labelled (^{2}H, ^{3}H, and ^{14}C) compounds for this purpose has been discussed. Coupling the use of AMS and isotopically labelled compounds for studies in neonatal research and care is also highlighted.

Keywords: Indazole; Wittig reaction; Estrogen receptors; SERM's; AMS; Cholestasis; Neonate

CARBON-14, DEUTERIUM, AND TRITIUM LABELING OF THE ALLYL SIDE CHAIN OF A HIGHLY SUBSTITUTED INDAZOLE

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Abstract: A selective estrogen receptor ligand [1-3] (**1**), which contains a highly substituted indazole core, was labeled with carbon-14, deuterium, and tritium to support drug metabolism, bioanalytical, and receptor binding studies, respectively. The allyl group side chain was the site for each of these labels. Carbon-14 labeling was achieved by use of the Wittig reaction to condense nonvolatile methyl[^{14}C]triphenylphosphonium iodide with an indazole acetaldehyde carbonyl group. Deuterium labeling was accomplished by alkylation of the indazole N(1) position with [D_5]-allyl bromide. Tritium labeling was possible by partial reduction of an acetylene analog of **1** with tritium gas using a poisoned catalyst.

Keywords: indazole; carbon-14; Wittig reaction; Lindlar catalyst; tritiation

Results and Discussion: Carbon-14 Labeling: A retrosynthetic dissection of the allyl group suggested that the Wittig reaction would be the best way to introduce a carbon-14 atom into **1** by condensing an indazole acetaldehyde with an ylid of nonvolatile methyl[^{14}C]triphenylphosphonium iodide. Alkylation [4,5] of dimethoxyphenol indazole (**2**) with excess bromoacetaldehyde diethyl acetal using sodium hydride in warm DMF gave the desired N(1) alkylation product **3** (23%) and also the N(2) alkylation side product (**4**) (71%) (see Scheme 1).

Hydrolysis of indazole acetaldehyde diethyl acetal (**3**) with p-toluenesulfonic acid in warm aqueous acetone, followed by removal of trace water by Dean-Starke azeotropic distillation with benzene, provided the target indazole acetaldehyde (**5**) needed for the Wittig reaction. The required phosphorus ylid was prepared from an excess (2.5 equivs) of methyl[^{14}C]tri-phenylphosphonium iodide [Amersham] using the dimsyl anion. After condensation of the ylid with aldehyde **5**, the methyl ethers in penultimate **6** were removed by treatment with boron tribromide (BBr_3) to provide [^{14}C]-**1** at a specific activity of 59 mCi/mmol in an overall 4.2% radiochemical yield.

Deuterium Labeling: A deuterated version of **1** was needed as a mass spectrometry internal standard, and [D_5]allyl bromide was the source of deuterium labels for this synthesis. By changing the base used to deprotonate indazole **2** from sodium hydride to lithium bis(trimethylsilyl)amide, a dramatic improvement in the yield of the desired N(1) alkylation product (**7**) [from 23% to 75%] occurred (see Scheme 2). Treatment of **7** with boron tribromide removed the two phenol methyl ether protecting groups to provide the final compound in modest yield.

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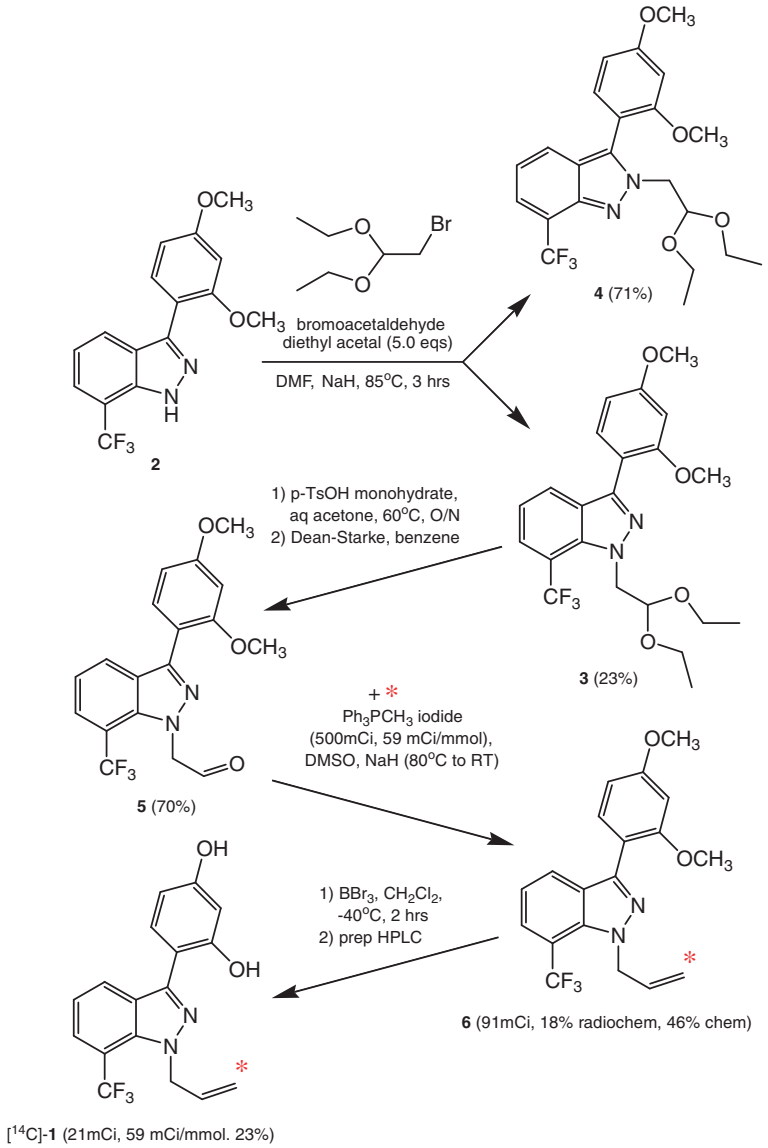
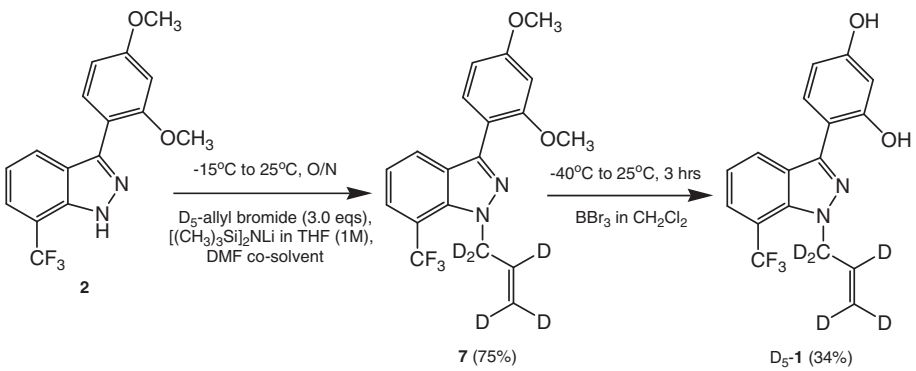


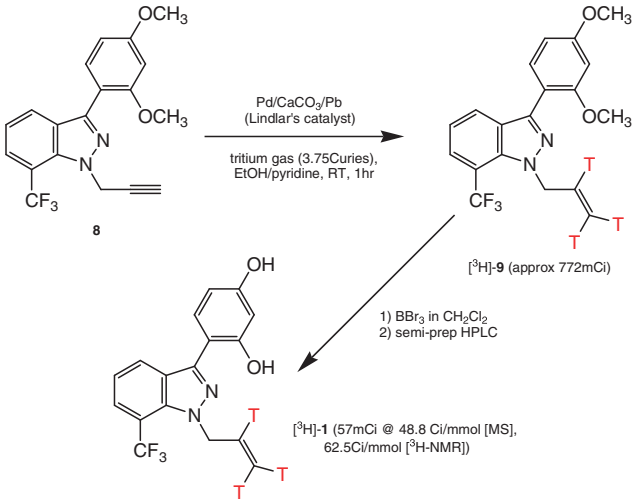
Scheme 1. Synthesis of [^{14}C]-**1** by Wittig reaction condensation of indazole acetaldehyde (**5**) with the ylid of methyl[^{14}C]triphenylphosphonium iodide [* = ^{14}C].



Scheme 2. Synthesis of [D_5]-**1** by alkylation with [D_5]-allyl bromide.

Tritium Labeling: A tritiated form of **1** was prepared to support receptor binding studies. A partial reduction of an acetylene analog **8** of the target compound using carrier-free tritium gas [TriSorber from IN/US Systems] in the presence of a poisoned catalyst was the best approach to prepare the tritiated allyl target compound **1**. Brief exposure of this acetylene analog **8** to approximately 3.75 Curies of tritium gas in the presence of Lindlar's catalyst [5% Pd on a calcium carbonate support, poisoned with lead] in a mixed

ethanol/pyridine solvent provided the crude tritiated allyl penultimate compound (**9**) (see Scheme 3). A small amount of the over-reduced side product containing a saturated propyl side chain was also observed in the crude reaction mixture.



Scheme 3. Synthesis of [^{3}H]-**1** by tritium gas reduction of an acetylene precursor (**8**) in the presence of a posoned catalyst (Lindlar's catalyst).

After a microscale deprotection of the two methyl ether protecting groups with boron tribromide, semi-v preparative HPLC purification provided 57mCi of the final [^{3}H]-**1** product. Mass spectrometry was used to determine a 48.8 Ci/mmol specific activity for [^{3}H]-**1**.

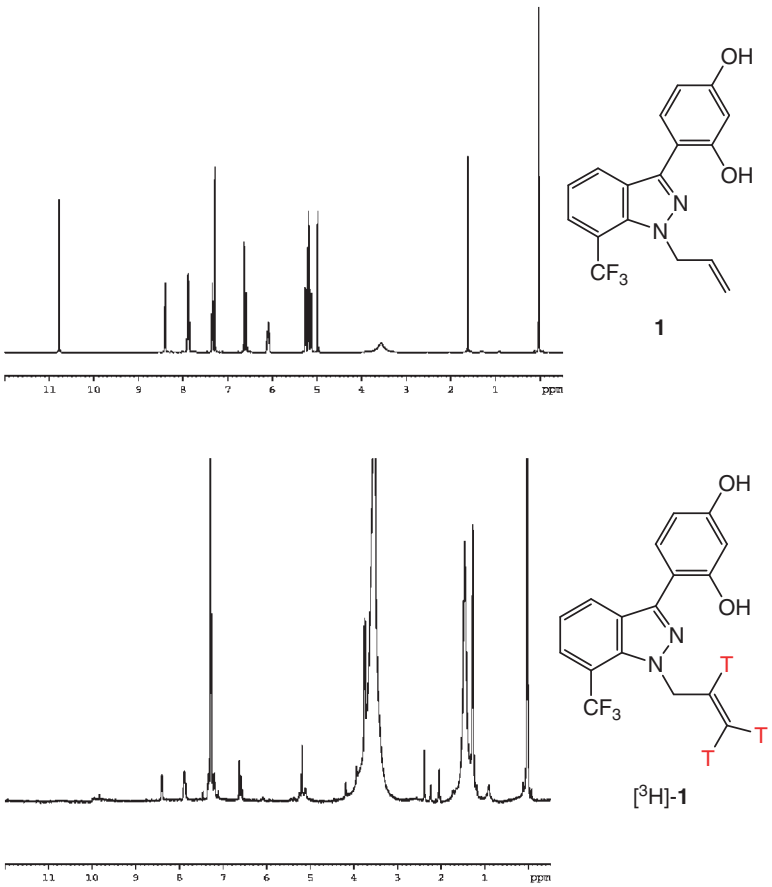


Figure 1. The 600 MHz ^{1}H-NMR spectra of unlabeled **1** (top) and [^{3}H]-**1** (bottom) showing the loss of proton signal intensities after tritiation (5.14 ppm, 5.26 ppm, and 6.09 ppm).

The ^{1}H NMR spectrum (in d_6-DMSO) of the unlabeled reference standard clearly shows the internal vinyl proton at 6.09 ppm, as well as a complex multiplet of the terminal vinyl geminal protons and the allyl methylene protons at approximately 5.2ppm (see Figure 1). The 600 MHz ^{1}H NMR spectrum of the tritiated product, in comparison, shows that the internal vinyl proton at 6.09ppm has been almost completely replaced by a triton. There is also a definite reduction in the complexity of the 5.2ppm multiplet confirming that most, but not

all, of the terminal geminal vinyl protons have been replaced with tritons. The ^{3}H NMR spectrum of [^{3}H]-**1** clearly shows the magnetic nonequivalence of the three different positions of the vinyl group occupied to different degrees by tritium atoms (see Figure 2). A comparison of the ^{3}H NMR spectrum of [^{3}H]-**1** with its ^{1}H NMR spectrum indicated that at least 97% of the internal vinyl position at 6.09ppm contains a tritium atom. The *cis* terminal vinyl position at 5.26ppm is approximately 80% occupied by tritium, and the *trans* terminal vinyl position at 5.14ppm is approximately 40% occupied by tritium. This uneven distribution of tritium in the terminal vinyl position indicates that there was predominately *cis*-addition of tritium across the acetylene during the reduction process, as expected. These ^{3}H NMR results indicate a tritium incorporation of 2.17 tritons per molecule corresponding to a specific activity of 62.5 Ci/mmol, somewhat greater than that determined by mass spectrometry.

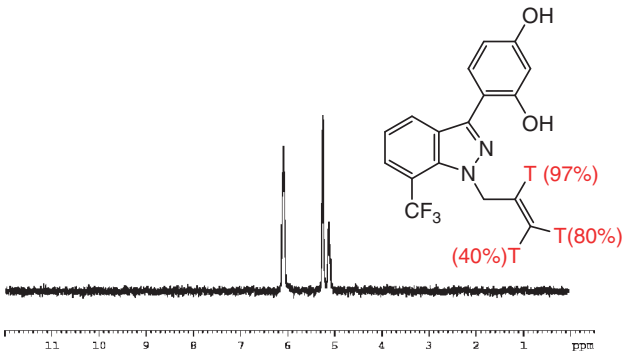


Figure 2. The 640 MHz ^{3}H-NMR spectrum of [^{3}H]-**1** showing the unequal incorporation of tritium atoms in the three terminal vinyl positions.

Acknowledgements: We thank Drs. Galina Vid and John Considine in the Wyeth Chemical Development group for supplying us with materials needed for the synthesis of the [^{14}C]-**1** and [D_5]-**1** compounds. Dr. Robert Steffan in the Wyeth Chemical and Screening Sciences group supplied us with the acetylene compound **8** used for the preparation of [^{3}H]-**1**. Dr. Melissa Lin in the Wyeth Discovery Analytical Support group provided the ^{1}H and ^{3}H NMR spectra of [^{3}H]-**1**. We also thank Dr. John Olszewski in the Wyeth Radiosynthesis group for his general technical assistance.

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THE SYNTHESIS AND METABOLISM OF AN ISOTOPICALLY LABELED SELECTIVE ESTROGEN RECEPTOR MODULATOR

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Abstract: Selective estrogen receptor modulators (SERMs) are an important class of compounds designed to suppress or inhibit estrogen function in postmenopausal women. The metabolism of these types of compounds may lead to reactive intermediates that may elicit adverse effects. Herein, we report the synthesis of a SERM that was differentially labeled with H-3 and C-14, for preclinical drug metabolism studies. We also describe the synthesis of deuterium labeled material for use as a bioanalytical standard.

Keywords: tritium; carbon-14; estrogen receptors and SERMs

Introduction: Estrogen plays an essential role in reproductive endocrinology and is important for supporting physiological well-being in women.[1] The biological effects of estrogen are mediated by estrogen receptors (ERs). Conversely, selective estrogen

receptor modulators (SERMs) are a class of compounds that bind to specific ERs in order to suppress or inhibit estrogen function. Selective suppression of ER activity has been shown to be beneficial in preventing age-related endocrine disorders. In order to provide pre-clinical drug metabolism support, the synthesis of isotopically labeled SERMs was required to facilitate ADME and covalent protein binding studies. This proceeding will describe the chemistry we utilized to access H-3, C-14 and stable isotope labeled SERMs (Figure 1).[2]

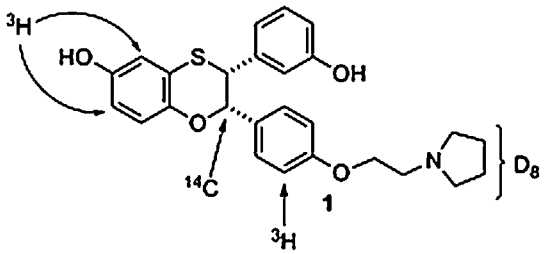
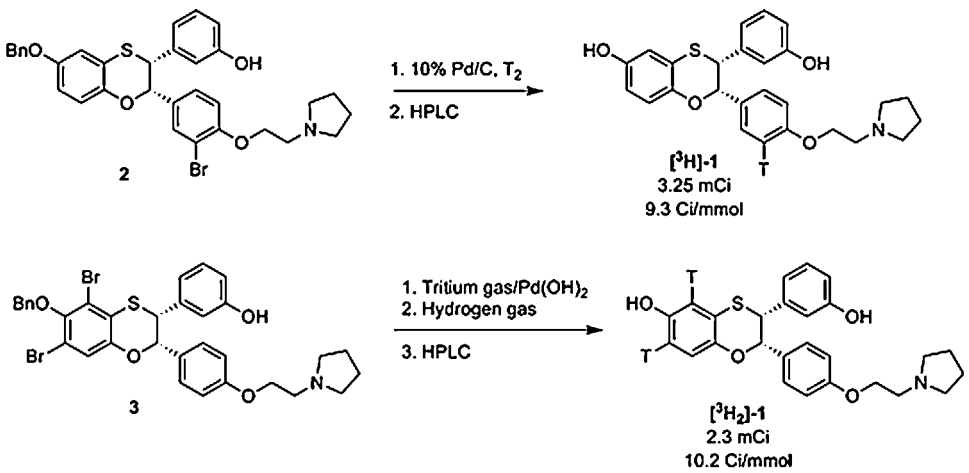


Figure 1. Labeling Strategy of SERMs Modulator (**1**).

Results and Discussion: The synthesis of **[^{3}H]** and **[$^3H_2$]-1** is summarized in Scheme 1. Benzyl protected **2** was treated with 10% palladium on carbon in the presence of tritium gas. Subsequent purification afforded 3.25 mCi of **[^{3}H]-1** in 99% radiochemical purity with a specific activity of 9.3 Ci/mmol. When this tracer was incubated with human liver microsomes and recombinant P450 3A4, it was observed that the portion containing the label, the 1-(2-phenoxyethy)pyrrolidine fragment, was lost.[3] As a result, we initiated the synthesis of **[$^3H_2$]-1** by treating dibromide **3** with tritium gas and palladium hydroxide (Pearlman's catalyst) followed by hydrogen gas. Purification by HPLC yielded 2.3 mCi of **[$^3H_2$]-1** in 99% radiochemical purity with a specific activity of 10.2 Ci/mmol. Although there were two bromides exchanged for the preparation of **[$^3H_2$]-1**, we did not observe a commensurate increase in specific activity.



Scheme 1

The C-14 labeled material was prepared by forming the Grignard reagent of benzyl bromide **4** and quenching with [^{14}C]carbon dioxide (250 mCi) to afford phenylacetic acid **5** in 95% yield. Acylation of acid **5**, in the presence of polyphosphoric acid, followed by deprotection with pyridine hydrochloride, gave bis-phenol **7** in 62% yield. Ketone **7** was differentially protected by first treating with methoxymethyl chloride followed by triisopropylsilyl chloride, to afford ketone **8** in 75% yield. Although the bis-MOM protected material was observed in 7% after the first step, it was easily removed by flash chromatography after silylation. Bromination of ketone **8** with phenyltrimethylammonium bromide perbromide followed by alkylation with thiol **10** gave thioketone **11** in good yield. The resulting adduct was cyclized to the *cis*-dihydrobenzoxathiin **12** with TFA and Et_3SiH as described[4] and subsequently resolved by chiral chromatography to afford enantiopure **12**. Treatment of phenol **12** with 1-(2-hydroxyethyl)pyrrolidine, under Mitsunobu conditions, afforded benzoxathiin **13** which was sequentially deprotected by debenzylation via transfer hydrogenation and desilylated with TBAF to afford **[^{14}C]-1**. The overall sequence required 14 radiochemical steps and afforded 7 mCi of **[^{14}C]-1** in 2.8% yield (Scheme 2).

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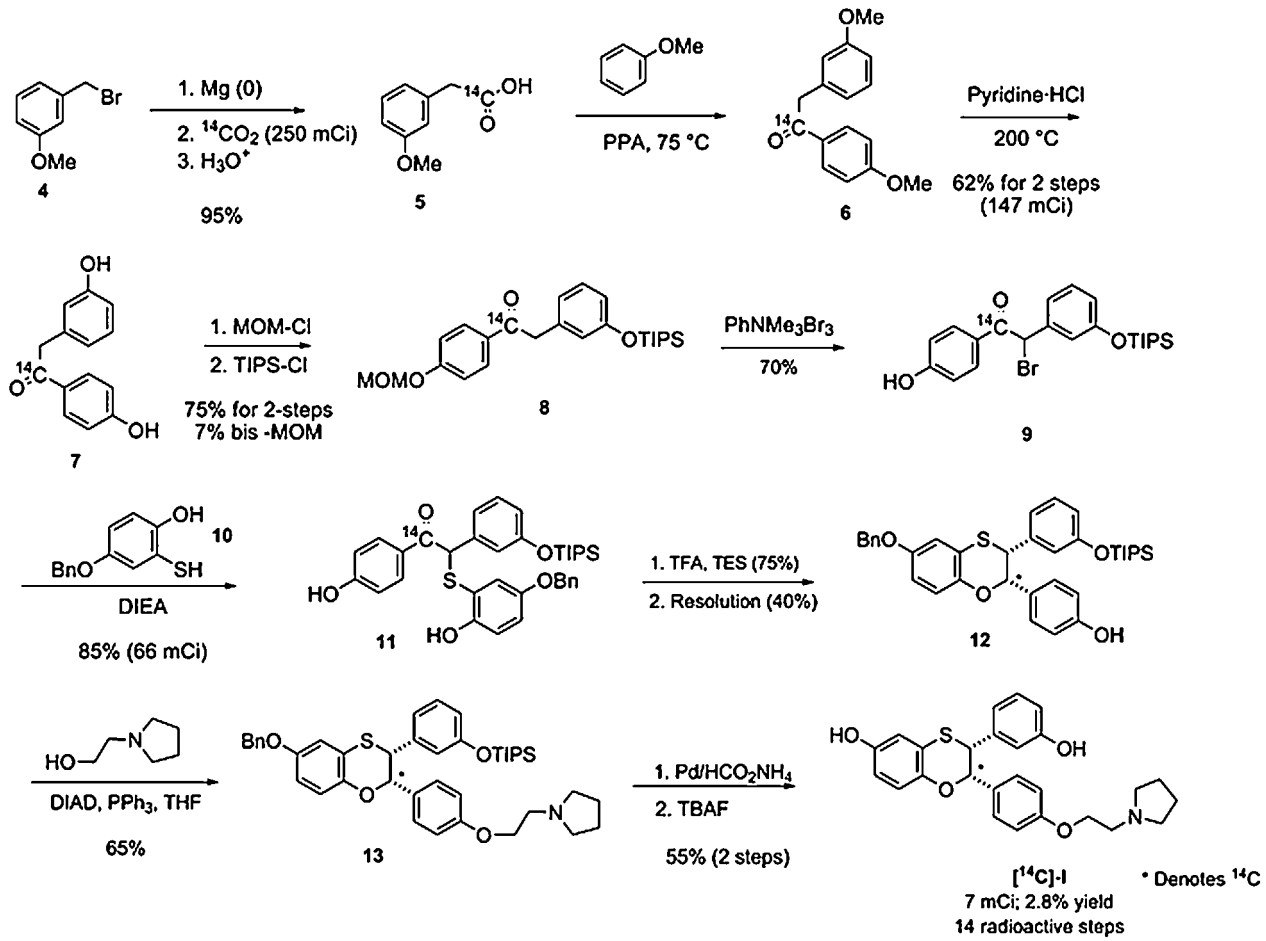
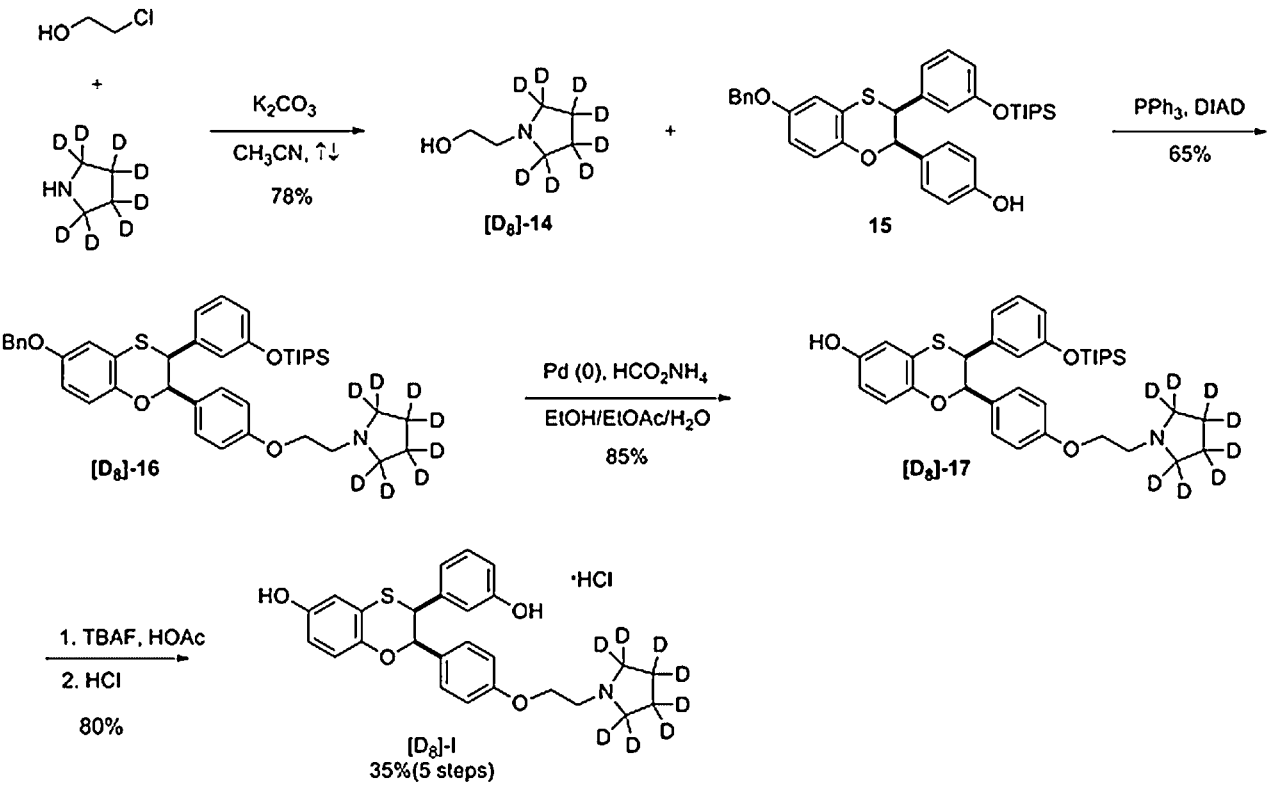


Scheme 2



Scheme 3

The synthesis of **[D_8]-1** was based on the route utilized for the **[^{14}C]-1** synthesis, by reacting **[D_8]**pyrrolidine with chloroethanol in refluxing acetonitrile, to afford alcohol **[D_8]-14**. The resulting alcohol was coupled with phenol **15** using standard Mitsunobu conditions to afford aryl ether **[D_8]-16**. Sequential deprotection by transfer hydrogenation followed by desilylation afforded **[D_8]-1** in 5 steps with an overall yield of 35%.

Conclusion: The chemical routes developed to synthesize **1** have been successfully adapted to introduce labels and provide four different tracers ([^{3}H], [$^3H_2$], [^{14}C] and [D_8]-**1**). These tracers were valuable tools for studying preclinical metabolism[3] and bioanalytical method development.

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APPLYING ^{14}C-AMS IN NEONATAL RESEARCH AND CARE

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Abstract: Ameliorating neonatal disease requires research and clinical tools that are sufficiently specific and sensitive to provide direct measurement of drug or nutrient ADME. Technologies used to study ADME in adults usually require larger sample volumes than can be collected from newborn infants. In this report we demonstrate a 'single-drop' analytical capacity for quantifying tracer labeled compounds in neonates using accelerator mass spectrometry (AMS) to quantify the concentration of sub-therapeutic doses of ^{14}C-labeled compounds in single drops (5 - 25 μl) of biological fluids, such as obtained from a heel prick drop of blood/ECF. We also discuss the methods and constraints in performing PK measurements and show data from three neonates dosed at 37, 120, and 370 Bq of ^{14}C labeled ursodiol to demonstrate the value of AMS in neonatal research.

Keywords: neonatal; cholestasis; ursodiol; ^{14}C; AMS

Introduction: Despite the high level of regulation for the development of drugs intended for adult populations and the publication of information specific to use and adverse effects coincident with approval, drugs are seldom tested in pediatric populations. As a result, there is a critical lack of relevant safety information and more specifically, assurance that a drug's metabolism and clearance paths are applicable across the range of physical and physiological development encompassed by neonatal and pediatric applications. Furthermore, drugs are seldom developed specifically for pediatric populations, let alone for neonatal populations.[1] Allometric scaling with large factors included for known uncertainties is used for projecting pediatric response to compounds studied in adults.[2] However, there is little supporting evidence for the assumptions made in scaling the distributions and pharmacodynamic effects of therapeutics or even potent nutrients to the neonatal population. This is because neonates have not yet developed full homeostatic balance in their biological systems and therefore have large inter-individual variabilities that can be dependent on gestational and postnatal age, as well as on birth weight, genetic factors, and possible disease exposures.[3,4].

Traditional series PK and PD studies of circulating compounds require hundreds of microliters of blood over multiple time points. The frequency and size of blood sample collections are naturally restricted in young children, infants, and, especially, in neonates. Doses of exploratory therapeutics must also be assuredly safe (low) for pediatric subjects in initial measures of the as yet unknown PK and PD properties and their variability, so that better estimates are available for studies involving therapeutic doses. The direct quantification of drugs, supplements, and nutrients in neonates is thus hampered by the need for samples of sufficient volume for typical analyses from very low exploratory doses. Desired data continuity is further limited by the ethical imperative to keep all procedures minimally invasive, especially in younger populations who are unable to personally provide informed consent.

Population pharmacokinetic modeling is one approach to obtaining drug development data from pediatric populations when the data is sparse from any one individual.[5] However, the large inter-individual variation and rapid maturation effects observed for

neonates blunts the power of the population based models and requires that studies have large cohorts. Several studies of parental consent to neonatal research report high recruitment,[6] and demonstrate good understanding of the informed-consent process,[7] but these studies were primarily from countries that have some form of national health care that may contribute to an active spirit of community obligation. Other studies report that consent for perinatal research was difficult to obtain.[8] Half of the randomized controlled trials surveyed in one study contained less than 40 subjects (less than 20 per arm), leading to low power in the conclusions, even when high rates of parent consent were reported.[6] Alternatives to population PK approaches are desirable due to this potential for low study power. Accelerator mass spectrometry (AMS) provides the high analytical sensitivity required for directly and accurately tracing isotope labeled compounds in individual neonatal subjects from safely low compound doses.

AMS Methods for Neonates: The suggestion that radiocarbon-labeled compounds be used in the clinic for the development of a drug or a diagnostic for pediatric and neonatal patients has seldom been brought forward. This is backed by a literature search covering the past 25 years that did not yield a single *in vivo* result using ^{14}C, despite the numerous reports of *in vitro* applications (e.g. cellular and *ex-vivo* tissue bioassays).[9] Quantitation of ^{14}C by any decay-counting analysis is inherently inefficient and requires comparatively large amounts of ^{14}C to yield meaningful results for *in vivo* clinical studies. [10] Typical human doses of ^{14}C for tracing purposes in adults heretofore are in the 4 MBq (108 μCi) range for decay counting of the radioisotope, and a 1:100 mass scaling suggests 40 kBq (1 μCi) would be required for a 1–2 kg neonate. This latter amount is 1000 times the natural level of ^{14}C in the 2 kg subject and therefore presents a barrier to consent based on real or exaggerated concerns regarding the risk of exposure when the welfare of neonates is considered.

AMS quantitation of ^{14}C has wide dynamic range and sufficient sensitivity to facilitate clinical studies based on administered ^{14}C doses that are similar in radioisotopic composition to subject's natural ^{14}C concentration.[10] A 2 kg neonate having an 80% body weight of water contains only 160 g of carbon, 1.2×10^{-12} of which (16 pmol) is ^{14}C with a radioactivity of 37 Bq (1 nCi). ^{14}C is not the dominant natural radioactive exposure within humans, and environmental sources (especially in aircraft at high altitude) are much larger. A 37 Bq dose, doubling the endogenous amount for the short duration of compound clearance, is appropriate as a starting point in determining the level of ^{14}C label required for tracing a compound in neonates with ^{14}C quantified by AMS. Parents are presented with a better understood risk from radiation exposure that is no greater than a few added days of life itself.

Neonates have plasma volumes of about 100 mL, and an adequate plasma sample for AMS analysis is less than 25 μl [10] that can be readily obtained by heel stick sampling of plasma and intercellular fluid. Fluid volumes collected by heel stick can vary without affecting the tracer concentration measurement, since AMS quantifies an isotopic ratio that internally normalizes the ^{14}C content in the sample against the carbon content of the sample. The sample volume collected is not required to obtain the comparative plasma concentrations of the samples. Plasma is homeostatically controlled in its carbon content, but, as argued above, this homeostasis is less dependable in neonates than in adults. An isotope dilution protocol using known spikes of exogenous ^{13}C is used with the AMS spectrometer to simultaneously quantify the carbon in selected samples while also quantifying the ^{14}C content in order to confirm the volumetric carbon concentration of heel stick fluid.

We performed an experiment to determine the optimum ^{14}C dose that could provide multi-day AMS tracing sensitivity in neonates. Five (5) pre-term neonates were recruited with their parents at the Loma Linda University Childrens Hospital (LLUCH), after their parents had provided full informed consent for the procedures. One (1) subject left the hospital before the end of the study, and one (1) subject was withdrawn by the parents before completion. The eligible population included infants that were more than two days of age and admitted to the LLUCH Neonatal Intensive Care Unit (NICU). Additionally, only infants with naso-gastric feeding tubes for direct dosing and indwelling venous catheters available for collection of blood samples were considered for participation in the study to avoid any additional invasive procedures. ^{14}C-ursodiol (ursodeoxycholic acid), a secondary bile acid making up 1 to 5% of an adult's bile acid pool, was dosed at 37, 122 and 370 Bq (1, 3.3, 10 nCi; 8, 24, 80 ng) with a 48 hour period between dosings to explore the ^{14}C required for adequate quantitation by AMS. No unlabeled ursodiol was added to the labeled compound, as will be done in a follow-on ascending therapeutic dose study in neonates with cholestasis. This was a pure 'nanotracer' experiment in which the kinetic response of the dose will depend on the pool of naturally circulating bile acids that easily dominate the tracer compound.

Neonatal cholestasis is a common affliction in infants admitted to NICUs. Approximately 10% of infants in the 84-bed LLUCH NICU are being treated for this disease at any given time. It is characterized by an insufficient flow of bile acids through the bile duct to the intestine due to cellular membrane damage from the strong detergent-like bile acids that are made more destructive by potentially low glutathione production in the developing liver. Ursodiol is a mild bile acid that is well tolerated and acts therapeutically by decreasing the concentration of more toxic bile acids, improving hepatic bile acid excretion, and reducing bile acid transit time through the liver. Ursodiol is insoluble in aqueous solutions and is solubilized in mixed micelles in the small intestine during absorption. Thus, although ursodiol is normally fully absorbed in the duodenum, ursodiol bioavailability may vary greatly in the intestine of the newborn since cholestasis causes decreased concentrations of bile and micelles.

Ursodiol is a naturally occurring endogenous compound, and only an isotopic label can make an equivalent analytical tracer that is quantitatively distinguishable. Stable isotopes are not suitable in this instance, because the dose of compound to the infant for adequate stable isotope sensitivity would have been physiologically untenable. The use of ^{14}C-ursodiol in humans was approved by the Radioactive Drug Research Committee of Loma Linda University. All procedures were approved by the Institutional Review Board of the Loma Linda University Children's Hospital.

Results: The initial blood draws from the five infants contained natural ^{14}C at the expected 1.061 ± 0.012 (1.1% CV) Modern. Modern is an international unit traceable to US NIST standard reference material, SRM 4990C. Among other equivalent definitions,[10] Modern equals an isotopic abundance of 1.176 pmol ^{14}C per mol C or 97.9 amol ^{14}C per mg C. The lower limit of quantitation (LLOQ) was taken as 6 times the standard deviation of this natural background measured in the infants' pre-dose blood samples, or 0.072 Modern (7 amol ^{14}C per mg C). Since the carbon concentration of plasma is approximately 40 mg/ml, this LLOQ was 0.28 pM for the ^{14}C label. The ursodiol was labeled at 0.8 mol ^{14}C per mol compound, or 50 Ci/mol, so that the resulting LLOQ for ursodiol was 0.35 pM.

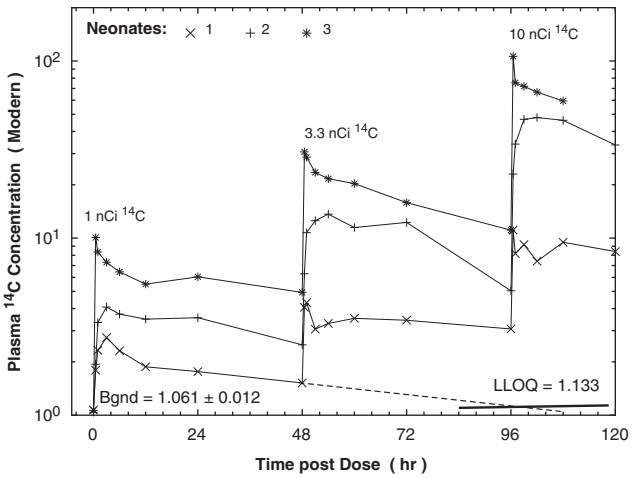

Figure 1. The ^{14}C concentration in Modern of 25 mL samples of plasma from three neonates, each dosed at 1, 3.3 and 10 nCi of ^{14}C-ursodiol with 48 hours between doses. Background natural ^{14}C was measured in an initial cohort of five subjects. The LLOQ represents 6 times the standard deviation in the background measurement.

Figure 1 clearly depicts the diverse response of neonates to the ^{14}C-ursodiol with observed values for C_{max} differing by a factor of 3 in the first dose and a factor of 10 in the third dose. As expected, a ^{14}C dose that was equal to a subject's natural ^{14}C provided sufficient signal for AMS quantitation, with plasma reaching factors of 3 to10 times the natural background ^{14}C concentration. This data represents dynamic ranges of 20:1 for the low responder to 120:1 for the high responder over the LLOQ (♯3: [10-1.07]Modern/ 0.072 Modern = 124). This LLOQ would be sufficient for tracing the extrapolated lowest dose (1 nCi) of the lowest responder (subject 1) for 96 hours post dose.

The third subject was observed to have a particularly rapid response, with 20% of the dose of this hydrophobic compound circulating and measurable in plasma within one half hour of gastric dosing. This was confirmed in the data for the higher doses that followed. An unusual gastric absorption must be assumed. The first subject showed not only low absorption of the compound, but also low enterohepatic (EH) recycling during the first dose which was seen in the other two (2) subjects with the rise in plasma ^{14}C concentration at 24 hours post dose administration. The first subject displayed signs of EH recycling during the second tracer exposure, 48 hours later. The 'nanodoses' used in this study should have no added pharmacodynamic effect on the biliary system, and the observed results may reflect the developing biology of the endogenous bile acids. Further discussion of the results or any conclusions about the behavior of neonatal biliary systems in response to small ursodiol doses is beyond the scope of this report which serves to introduce the concept of AMS tracing ^{14}C compounds in neonates.

Conclusion: We report what we believe to be the first tracing of a ^{14}C-labeled compound in neonatal subjects using ^{14}C doses that were commensurate with natural levels and required only 25 μl of plasma per data point. This low specimen volume was taken from indwelling catheters for this study, but would be also readily available with less invasive collection methods, such as heel sticks. AMS sensitivity was sufficient to trace the labeled compound for at least 4 days, even in poorly absorbing subjects. The radiation exposure from this ^{14}C dosing was the equivalent of a few days of life averaged over the entire body. This data demonstrates that AMS tracing is an effective, safe, and non-invasive approach to studying drug and nutrients in pediatric subjects, and in the extreme case of neonates no older than a few days. This data supports the application of AMS towards the development of: 1) clinical tools to diagnose neonatal pathologies based on metabolic/physiological imbalances; 2) clinical tools to directly observe responses of neonates to administered drugs; 3) research tools to facilitate the improved clinical research and development of diverse drugs and biomarkers for neonatal patients.

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