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Abstract: Diverse topics, with a central theme are discussed. On the one hand, the preparation of an anti-epidermal growth factor MAB, labelled with ^{177}Lu is detailed. In addition, traditional syntheses of isotopically labelled small molecules are also highlighted.

Keywords: Epidermal growth factor; lutetium; MLN3897; C-14; Apixaban; Factor Xa; deuterium

LABELLING OF ANTI-EPIDERMAL GROWTH FACTOR MONOCLONAL ANTIBODY WITH ^{177}LU:RADIOCHEMICAL AND BIOLOGICAL EVALUATION

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Abstract: This study aims to evaluate the synthesis parameters of ^{177}Lu-anti-EGF-Mab using a macrocyclic ligand, DOTAM (2,2′,2″,2‴-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetamide) and its in vivo biodistribution in animal models. DOTAM-EGF was synthesized in high yield at room temperature in 0.1 M $NaHCO_3$ pH 8.2 using a mixture of DOTAM/anti-EGF-Mab in nonstoichiometric ratio. The complex was then labeled with ^{177}Lu (15 mCi $^{177}LuCl_3$, pH 4) with more than 95% labeling efficiency. Biodistribution evaluation revealed a major accumulation in periferal tumor tissues (up to 12.49% ID/g) at 24 h after i.v. injection while the tumor mass uptaked 7 times less activity. The liver, kidneys, and small intestine showed a moderate took up, the mean value was 3.4 ID/g in the first two hours post injection following a decreasing curve up to 0.5% ID/g at 72 h post injection. Based of the preliminary results of this study ^{177}Lu-(DOTAM)anti-EGF-Mab will be tested as a new radiopharmaceutical for epidermal malignancies.

Keywords: epidermal growth factor; lutetium-177; targeted radiotherapy

Introduction: One of the latest approaches in cancer therapy implies the use of a high linear energy transfer, nonpenetrating radiation specifically delivered at cellular level. Receptor-mediated radiotherapy (also called targeted radiotherapy, TRT) relies on the use of a receptor specific ligand to transport a radionuclide to tumor cells that overexpress the target receptor. The selection of the ligand is based on its ability to selectively target cancer cells. The therapeutic radiation is delivered to tumors while minimizing radiation exposure to normal tissues. In this concept, the radioligand should be rapidly cleared from circulation; the chelate should be stable under physiological conditions; the tumor cells should express a sufficiently high density of receptors and a long biological residence time of radioactivity is needed. The receptor internalization is the basic process that sustains the selectivity and therapeutic efficacy of targeted radiotherapy while the radioactive metabolites should remain trapped in the cell. Researchers are investigating both the agents that seek out specific tumor cells for treatment and the radioisotope payload that delivers the radiation.

The EGFR (Epidermal Growth Factor Receptor) is a validated anticancer target. The physiological roles of EGFR ensure epidermal renewal and integrity. Alterations in the EGFR family are involved in the development of cancer leading to the stimulation of tumor growth, protection from apoptosis, angiogenesis, and the formation of metastases[1]. Increased EGFR expression is an adverse prognostic feature of

many cancers. Epidermal growth factor receptor inhibitors have been developed in recent years as therapeutic molecules directed against cancer with an overall dominating effect of inducing growth arrest and terminal differentiation of the keratinocytes in the basal layers.

A major decision to make for TRT is the radionuclide, where the nonpenetrating emissions are relevant for therapy. The suitability of a radionuclide for TRT depends on its physical and chemical properties, its fate after antibody metabolism in vivo and the nature of the radiation, such as low or high linear energy transfer. One radioisotope that may help create the first successful radiopharmaceutical for solid tumors is lutetium-177 (Lu-177); it emits a low beta energy, which reduces radiation side effects and produces a tissue-penetration range appropriate for small tumors.

Aim: Starting from the recently developed agents for targeted molecular imaging we tried to extrapolate the use of the epidermal growth factor as biological carrier for targeted radiotherapy, aiming to develop a radiopharmaceutical for targeted radiotherapy based on anti-epidermal growth factor antibody, as a carrier vehicle for non-penetrating radiation delivery. The epidermal growth factor monoclonal antibodby (anti-EGF-Mab) is an attractive targeting agent due to its low molecular weight (6 kDa) and high affinity for EGFR[2,3].

We have labeled the EGF with beta emitter Lu-177 (half life 6.71 d, $E_{\beta_{max}}$496 keV, Eγ 208 keV, short-range tissue penetration, mean range 670 μm). Lu-177's low beta energy together with long half-life and high specificity make it a promising isotope for radiopharmaceutical treatment of solid tumors[4] and also for micro metastatic disease[5].

The study aims to evaluate the synthesis parameters of the ^{177}Lu-antiEGF-Mab using a macrocyclic ligand, DOTAM (2,2′,2″,2‴-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetamide) and its in vivo biodistribution in normal and HRS1 tumor bearing animal models.

Materials and Methods: The labeling of anti-EGF-Mab with Lu-177

An indirect radiolabeling procedure was employed. DOTAM (Figure 1) was used as chelating agent for the metal, which forms an in vivo stable complex with lutetium. The labeling procedure was optimized by varying the reaction parameters such as: temperature, reaction time, molar ratio (DOTAM to mAb).

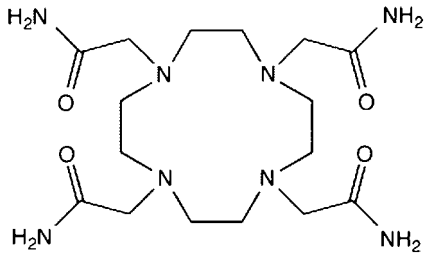

Figure 1. DOTAM (2,2′,2″,2‴-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetamide).

DOTAM was attached to the mAb's structure in the inactive part not to alter the antibody biological specificity. The coupling of DOTAM with EGF mAb was done in the first step and then the Lu was introduced in the construct.

First step: 1 mg EGF-mAb was added to 1.93 mg DOTAM in sodium carbonate buffer $NaHCO_3$ 0.1 M (pH = 8.2). After 24 h incubation at room temperature the solution was dialyzed in acetate buffer (pH = 5.5). The pH of DOTAM-EGF conjugate was 5.5.

Second step: 14 mCi $^{177}LuCl_3$ in 0.2 mL was added to the DOTAM-EGF conjugate. The probe was incubated at 45°C for 1 h.

Radiolabeling yield and radiochemical purity of ^{177}Lu-DOTAM–EGF were tested by PC (Whatman 1) and ascending instant thin-layer chromatography (ITLC) with silicagel-coated fiberglass sheets 20 cm length (Polygram SIL G, Macherey-Nagel, Germany). The R_f's of free lutetium, lutetium chloride and ^{177}Lu-DOTAM-EGF are presented in Table 1. The radiochemical purity was calculated by subtraction of percents of impurities determined by both methods.

The probes intended for biological studies were diluted with saline (NaCl 0.9%) to 8 mL, according to the radioactive concentration and biological concentration to be administered.

Table 1. Rf of the radiochemical species existing in the solution

Solvent	Method	^{177}Lu-DOTAM-EGF	$^{177}LuCl_3$	^{177}Lu free
Sodium citrate 0.1 M, pH = 5	PC	Rf = 0.62–0.70	Rf = 1.0	—
Ammonium acetate 10%:Me-ol (30:70)	ITLC	Rf = 0.76–0.85	—	Rf = 0.00–0.16

Biodistribution studies: The experimental model used was tumor bearing young male rats from Wistar line (*rattus norvegicus* albinos variety, rodentia, mammalia), 200–250 g. The animals were kept in cages under ambient temperature and humidity, receiving commercial ration and water *ad libitum*.

Studies regarding biodistribution of Lu-DOTAM-EGF were done using 3 animals/each time point. The animals were anesthetized with a mixture (0.2 mL/animal) containing 0.15 mL ketamine 10% and 0.05 mL acepromazine (Calmivet), injection using an insulin type syringe and needle in the peritoneal cavity. They received ^{177}Lu-DOTAM-EGF by i.v. injection in the tail vein and then they were sacrificed at 2 h, 4 h, 24 h, 48 h, 72 h p.i., expressive tissues were removed and their radioactivity were measured. The results are expressed as %ID/organ.

Evaluation of the competitive binding of ^{177}Lu-DOTAM-EGF to the EGFR: In the therapy protocol we propose to use stem cells as support for the radiation therapy. In this context, we have tested the competitive binding of the radiolabelled antibody to its receptors on a pancreatic cancer cell line and also to a mesenchimal stem cell line.

2 experiment plaques containing 4×10^5 pancreatic tumor cells (labeled P1 respectively P2) and 2 plaques containing 4×10^5 mesenchimal stem cells (labeled P3 respectively P4) were prepared. To each probe 0.5 mL was added containing decreasing

Journal of
Labelled Compounds and
Radiopharmaceuticals

concentrations of mAb (10^2, 10, 1, 10^{-1}, 10^{-2}, 10^{-3} μg EGF) and constant radioactivity (396.000 cpm in 10 μL) ^{177}Lu-DOTAM-EGF. The cells were incubated at 37°C for 2 h (P1 and P3) and 4 h (P2 and P4). The cells were washed 2 times with 1 mL PBS and water. Cells were detached and dissolved in 400 μL NaOH 2N/alveola, 30 min, at 25°C. The Eppendorf tubes were counted (Raytest, ^{177}Lu calibrated).

Results and Discussion: Synthesis of ^{177}Lu-DOTAM-EGF

The DOTAM-EGF complex was synthesized in high yield at room temperature in 0.1 M $NaHCO_3$ pH 8.2, using a mixture of DOTAM/anti-EGF-Mab in nonstoichiometric ratio. The complex was then labeled with ^{177}Lu (15 mCi $^{177}LuCl_3$, pH 4) with more than 95% labeling efficiency; the specific activity was 1400 mCi/mg. The RCP of ^{177}Lu-DOTAM-EGF was 95–96% (Figure 2).

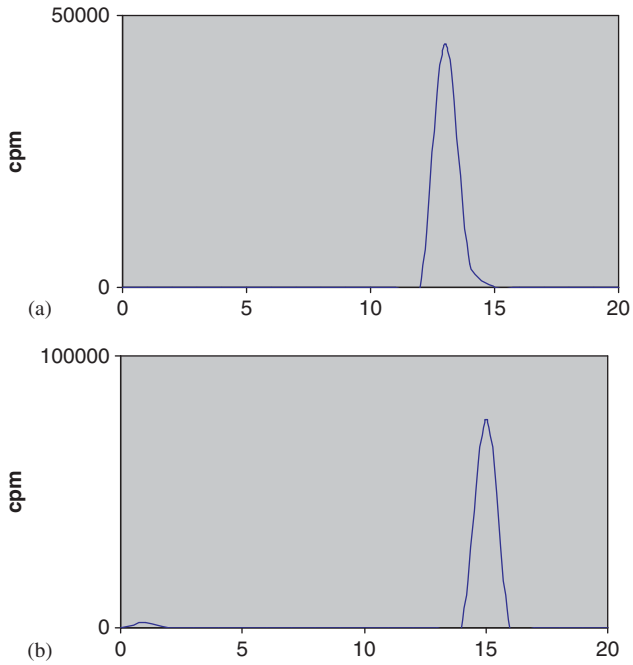

Figure 2. Radiochromatograms of ^{177}Lu-DOTAM-EGF using (a) PC and (b) ITLC.

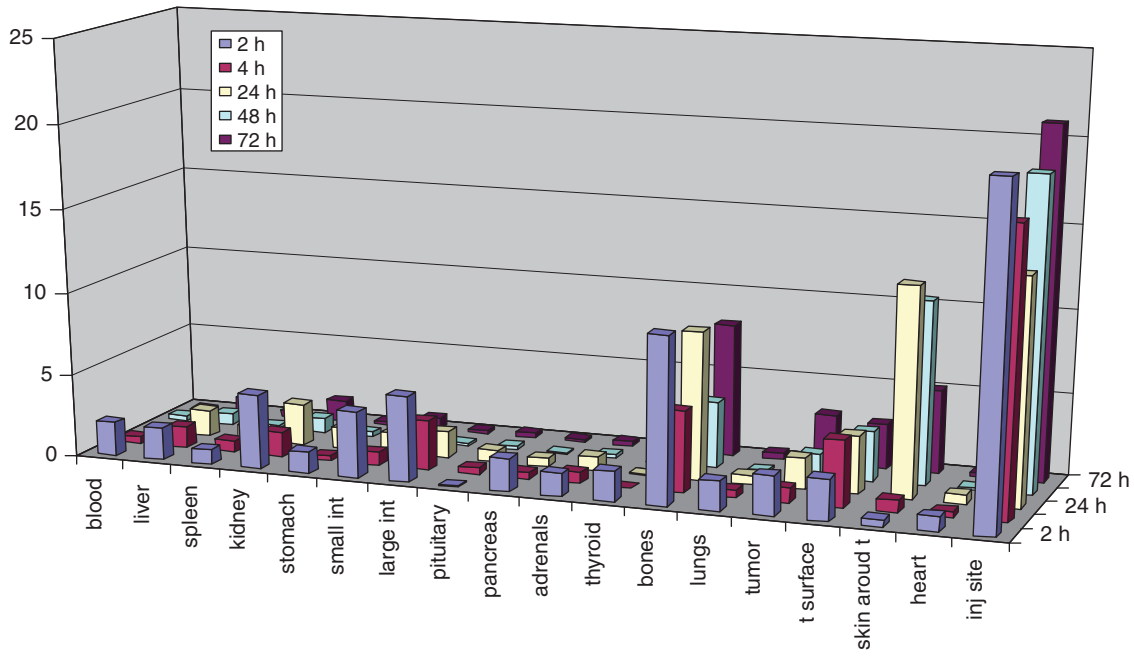

Figure 3. Biodistribution of ^{177}Lu-DOTAM-EGF in tumor bearing animal model.

Biodistribution of *^{177}Lu-DOTAM-EGF*: Biodistribution studies revealed important aspects of ^{177}Lu-DOTAM-EGF in vivo behaviour (Figure 3).

- About 20% of the ID remains at the injection site, in the tail; fast blood clearance was observed (2–4 h);
- Small liver uptake but important activity was found in the stomach and intestines; this activity washed out with time;

- Other glands also show some uptake (up to 3% in pancreas and thyroid) at 2 h pi;
- Excretion is done mainly by kidney which receive about 4% of the ID;
- The tumor received not more than 3% ID but some regions of the tumor, like the tumor surface and skin around the melanoma received 4 and 12% of the ID respectively, which shows very good specificity of the product to the newly created and active tumor cells.

We observed important bone uptake, up to 9% ID, and we asked if it is free ^{177}Lu as a radiochemical impurity or decomposition is due to the in vivo metabolism process. We have also checked the bone uptake of lutetium chloride, as the free Lu specifically bonds to this tissue. In the case of $^{177}LuCl_3$, the radioactivity is accumulated up to 24 h followed by slow and constant lost. The bone accumulation pattern after ^{177}Lu-DOTA-EGF administration is similar to a pump, with alternative increasing and decreasing segments. As the patterns of the bone uptake are different, we can conclude that the radioactivity accumulated in bones is released continuously by the in vivo breakage of the radiolabeled antibody.

In vitro experiments: The preliminary in vitro experiments show that ^{177}Lu-DOTAM-EGF binds to EGFR on the cell surface. The binding stability and internalization depend on cell type (Figure 4).

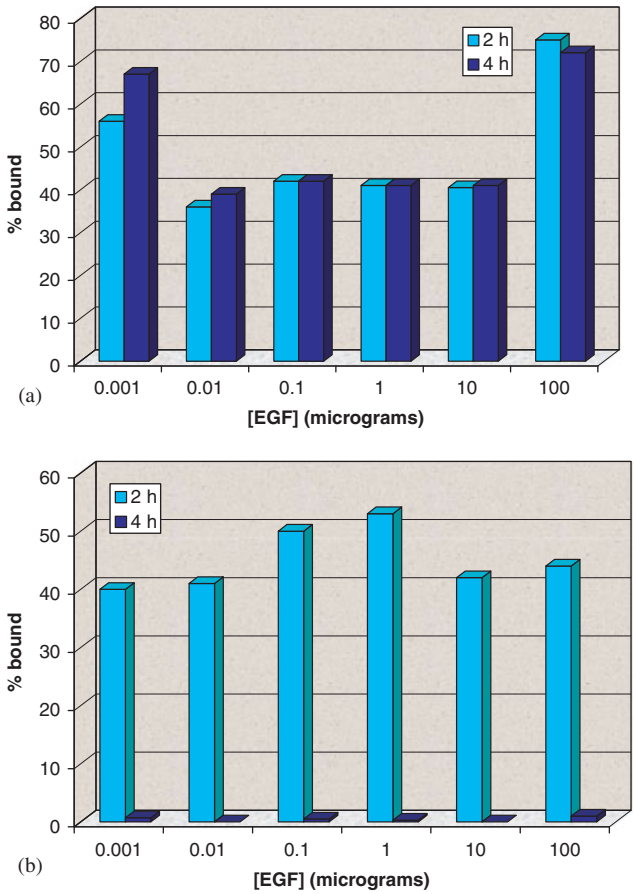

Figure 4. Binding of ^{177}Lu-DOTAM-EGF to: (a) pancreatic tumor cells and (b) mesenchimal stem cells at 2 and 4 h.

The bioaffinity of ^{177}Lu-DOTAM-EGF to tumor cells is higher than the bioaffinity to stem cells at 2 h. At 4 h, the ^{177}Lu-DOTAM-EGF was internalized in cancer cells while the radiolabeled antibody was washed from stem cells.

In conclusion, the high accumulation of ^{177}Lu-DOTAM-EGF in tumor surface and skin around the tumor (up to 12% ID) at 24 h after i.v. injection shows a very good specificity of the product to the newly created and active tumor cells. The stem cells support targeted radiotherapy since the stem cells should release ^{177}Lu-DOTAM-EGF continuously up to 4 h after its in vivo administration.^{177}Lu-(DOTAM)-anti-EGF-Mab can be considered a potential new radiopharmaceutical for epithelial malignancies.

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The experiments comply with the current Romanian laws.

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Journal of Labelled Compounds and Radiopharmaceuticals

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SYNTHESES OF ISOTOPICALLY LABELED VERSIONS AND MAJOR METABOLITES OF CCR1 ANTAGONIST MLN3897

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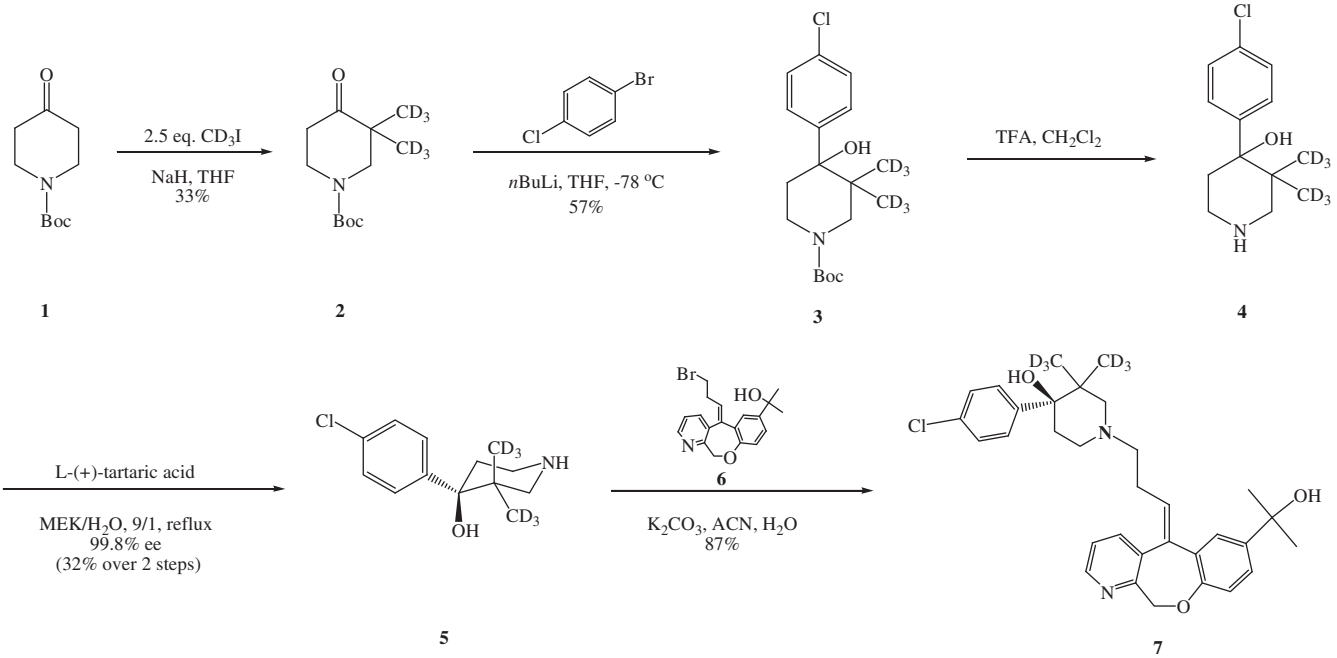
Abstract: MLN3897 citrate is a novel CCR1 antagonist that was taken into development for treatment of chronic inflammatory diseases such as multiple sclerosis and rheumatoid arthritis. Non-clinical data had indicated extensive metabolism of MLN3897 via *N*-dealkylation into two halves. The in vivo disposition of [^{14}C]-MLN3897 in humans was therefore investigated with the [^{14}C]-labeled drug candidate in either the tricyclic (**13**) or the chlorophenyl (**18**) moiety. The use of two separate [^{14}C]-radiolabels of MLN3897 facilitates identification and quantitation of metabolites resulting from each half of the molecule. The first preparation began with commercially available [1-^{14}C]-acetyl chloride via Friedel-Crafts acylation following the patented procedure. The second synthetic route was designed to incorporate the radioactive label as late as possible. In this key step freshly generated [^{14}C]-*p*-chlorophenyllithium reacted with ketone **15** to provide a racemic mixture that was later resolved by preparative chiral HPLC. Detailed syntheses of the two radiolabeled versions and significant metabolites will be described.

Keywords: [^{14}C]-MLN3897; [1-^{14}C]-acetyl chloride; [^{14}C]-*p*-chlorophenyllithium; [D_6]-MLN3897; MLN3897 metabolites

Introduction: MLN3897 citrate, (*S*)-4-(4-chloro-phenyl)-1-{3-[7-(1-hydroxy-1-methyl-ethyl)-11*H*-10-oxa-1-aza-dibenzo[a,d]cyclohepten-(5*E*)-ylidene]-propyl}-3,3-dimethyl-piperidin-4-ol, is an orally active, small molecule antagonist of C-C Chemokine Receptor-1 (CCR1)[1–3]. CCR1 is found on the surface of various cells in the immune system and is involved in a number of inflammatory diseases. Non-clinical data had indicated that MLN3897 is extensively metabolized, initially by an oxidative *N*-dealkylation process into two halves. Labeling the tricyclic (**13**) or the chlorophenyl (**18**) moiety with carbon-14 was required to address in vivo disposition of MLN3897. The use of two labeled versions of MLN3897 facilitates identification and quantitation of metabolites resulting from each half of the molecule. The stable isotope labeled version of MLN3897 (**7**) was also required as an internal standard for mass spectrometry based bio-analytical assays.

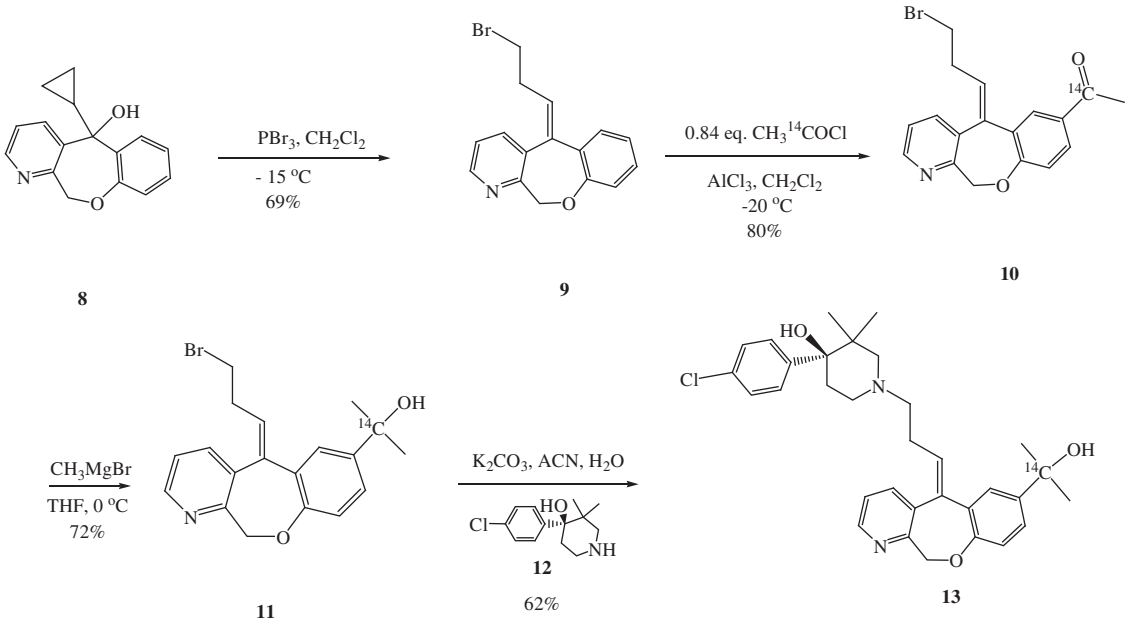
Results and Discussion: Synthesis of [D_6]-MLN3897 (7)

Based on the synthesis of unlabeled compound reported earlier[4,5], [D_6]-MLN3897 was prepared from inexpensive deuterated iodomethane. Since MLN3897 contains a chlorine atom, a labeled version that has at least 5 amu higher than the unlabeled version is required to ensure complete separation of labeled and unlabeled molecular ion clusters during mass spectrometric assays. The synthesis commenced from commercially available *N*-Boc-piperidone (**1**) which was treated with a slight excess of deuterated iodomethane leading to a mixture of mono-, bis- and tri-alkylated products. The desired bis-alkylated product (**2**) was isolated by trituration with petroleum ether, then condensed with a substituted organometallic reagent. Subsequent Boc-deprotection and classical resolution from L-(+)-tartaric acid provided the [D_6]-(*S*)-4-(4-chloro-phenyl)-3,3-dimethyl-piperidin-4-ol (**4**) with high enantiomeric purity (>99%). The tricyclic bromide **6** was elaborated from commercially available 7*H*-furo[3,4-*b*]-pyridin-5-one in 5 steps[4,5]. Final coupling with the bromide provided [D_6]-MLN3897 (**7**) with >98% isotopic abundance (Scheme 1).



Scheme 1. Synthesis of [D_6]-MLN3897.

Synthesis of tricyclic [^{14}C]-MLN3897 (13): [^{14}C]-MLN3897 was labeled on the tricyclic portion of the molecule in three synthetic steps according to the patented methodology as shown in Scheme 2[4,5]. Initial treatment of **8** with phosphorous tribromide at −15°C afforded the (*E*)-isomer of the 3-bromopropylidene derivative (**9**) in 98% isomeric purity after flash chromatographic purification. The homoallylic bromide was susbsequently acylated with [1-^{14}C]-acetyl chloride under Friedel-Crafts conditions to provide (*E*)-1-[5-(3-bromopropylidene)-5,11-dihydro-10-oxa-1-aza dibenzo[a,d]cyclohepten-7-yl]-[1-^{14}C]-ethanone (**10**) in 80% yield. Grignard addition of methyl magnesium bromide completed the synthesis of **11**, which was subsequently alkylated with the chiral piperidinol (**12**). The desired product **13** was isolated by flash chromatographic purification. Final treatment with citric acid and recrystallization from acetone provided tricyclic [^{14}C]-MLN3897 as the citrate salt.



Scheme 2. Synthesis of tricyclic [^{14}C]-MLN3897.

Synthesis of chlorophenyl [^{14}C]-MLN3897 (18): The radiolabeling of the northern part of MLN3897 molecule was designed to incorporate the carbon-14 label at a later stage in the synthetic sequence. In order to minimize the radiochemical steps we explored the coupling of the keto-alcohol **15** with a labeled aryl halide (Table 1). This would create a new chiral center that can also be resolved by utilization of chiral HPLC methods. The key precursor **15** required for the labeling step was prepared from commercial *N*-Boc-piperidone (**1**) which was subsequently bis-alkylated, deprotected, and condensed with the tricyclic bromide **6.**

Initial condensation reactions of ketone **15** were attempted with commercially available *p*-chlorophenyl magnesium bromide. Unfortunately no product was detected in an ethereal solvent (Et_2O or THF) either at room temperature or under reflux conditions for a prolonged period of time (1 to 5 days)[6]. It was believed that the "bulky" ketone **15** may require additional time and large excess of Grignard reagent for advancing the reaction. This led us to the development of another methodology that is based on the use of *p*-chlorophenyllithium. Attempts made at reactions of *p*-chlorophenyllithium with ketone **15** are summarized in Table 1. Addition of slight excess of ketone **15** to aryllithium species (generated from *p*-chloroiodobenzene and *t*-butyllithium in the presence of LiBr salt) did not result in formation of the desired product. Use of two equivalents of *p*-chlorophenyllithium at −78°C

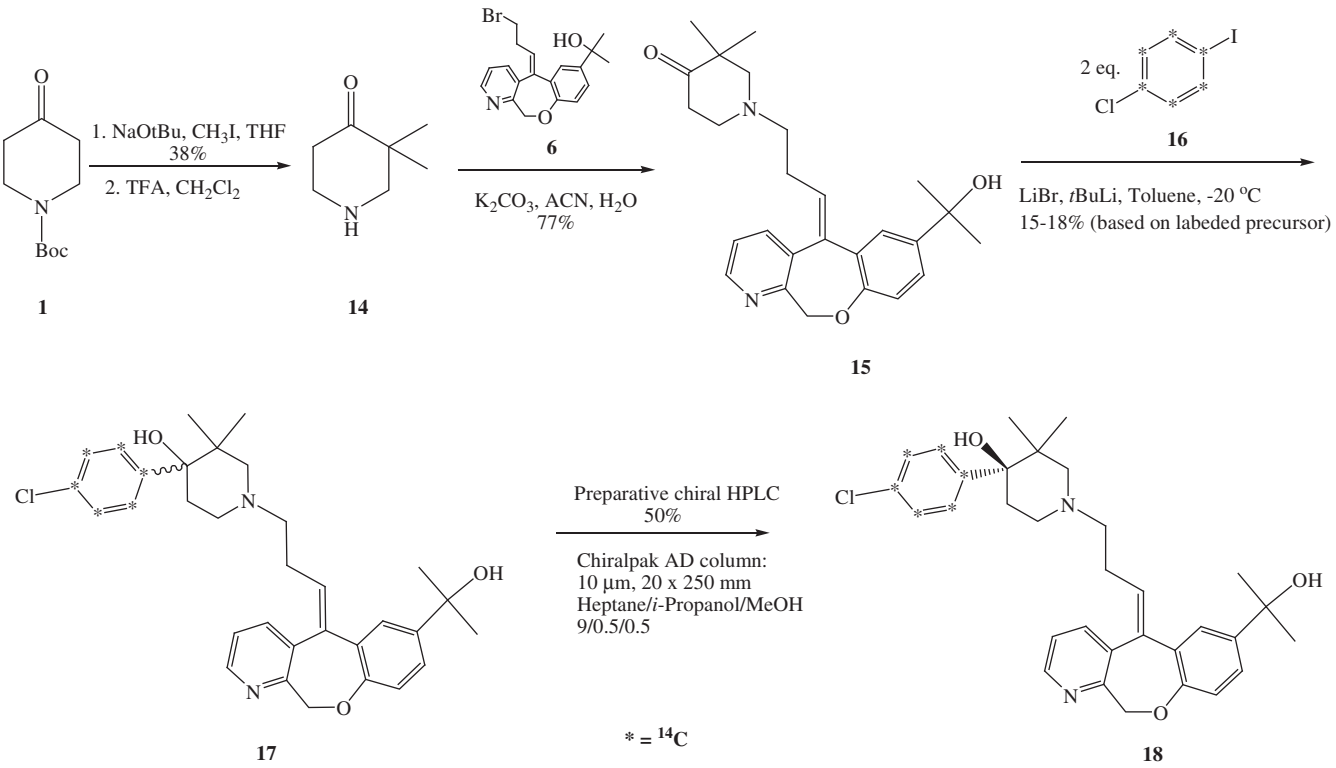
Table 1. Optimization of chlorophenyl [^{14}C]-MLN3897 synthesis

Ketone (eq.)	*p*-Cl-I-benzene (eq.)	LiBr (eq.)	Solvent	Temp.(°C)	Rxn time (h)	Yield (%)
1.2	1	2.35	Ether	−78	6	0
1.2	1	2.35	THF	−78	6	0
1	2	2	Ether	−78	6	11
1	2	2	Toluene	−78	6	14
1	2	2	Toluene	−50	6	30
1	2	2	Toluene	−40	6	17–33
1	2	2	Toluene	−10	6	31(impurities)
1	2	2	Ether/Toluene 1/1	−30	6	impurities

Journal of Labelled Compounds and Radiopharmaceuticals

for 6 h provided the product in 11% yield[7]. Slight improvement was noted with the use of non-polar solvent toluene[8]. The best yield was observed when this reaction was conducted at −40°C. Many impurities were also formed when using solvents of intermediate polarity (toluene/diethyl ether mixture).

Scheme 3 illustrates the final synthetic route to chlorophenyl [^{14}C]-MLN3897. Using the best conditions described above, 2 equivalents of freshly generated [^{14}C]-*p*-chlorophenyllithium (from *p*-chloroiodo[U-^{14}C]benzene **16** and *tert*-butyllithium) reacted with **15** to provide a racemic mixture **17**. The desired (*S*)-isomer **18** isolated by preparative chiral HPLC in >99% enantiomeric excess (ee) was converted to the citrate salt.

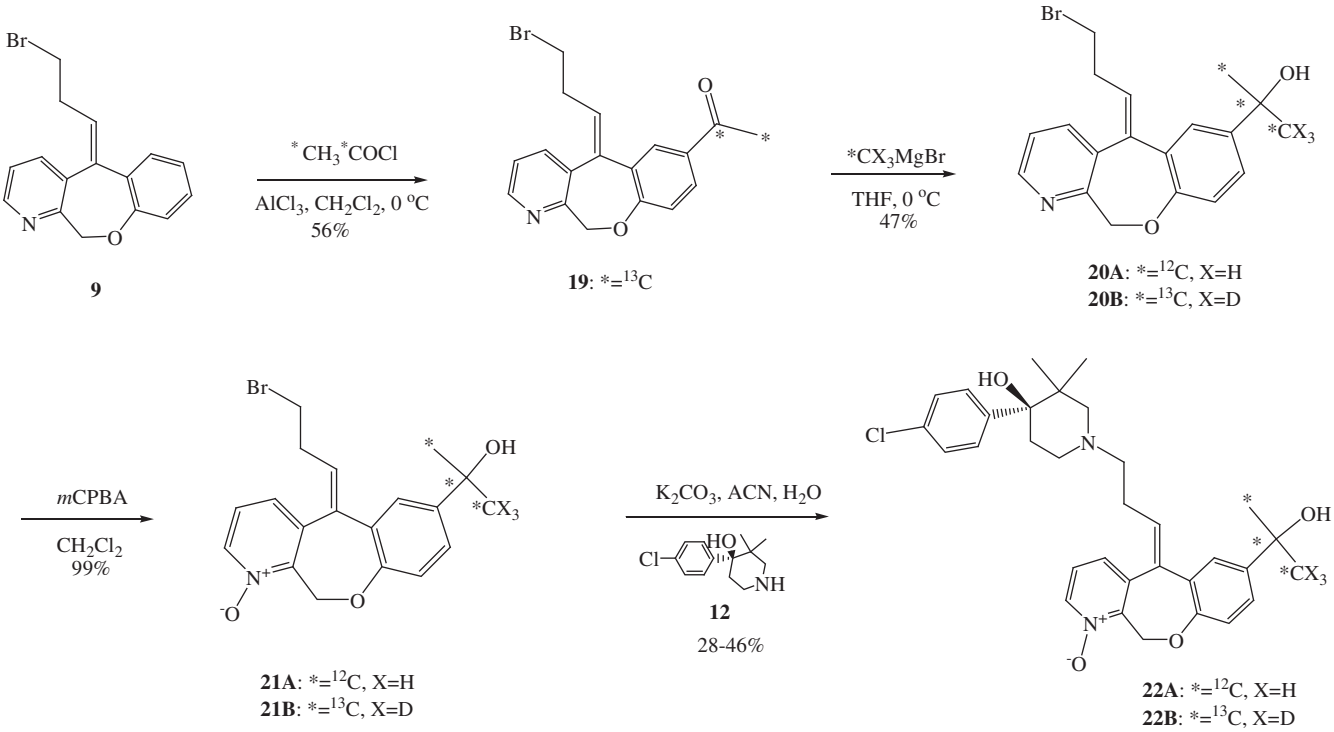


Scheme 3. Synthesis of chlorophenyl [^{14}C]-MLN3897.

Syntheses of major MLN3897 metabolites: Apart from MLN3897, the predominant metabolites in systemic circulation were the N-oxides of MLN3897 (M28) and of the carboxylic acid (M11), carboxylic acid (M19), and 4-(4-chlorophenyl)-4-hydroxy-3, 3-dimethylpiperidin-2-one (M44)[9]. These metabolites (M28, M11, M19 and M44) were synthesized to provide positive identification. The labeled internal standard of M28 was also prepared to assist in monitoring of this metabolite in clinical trials.

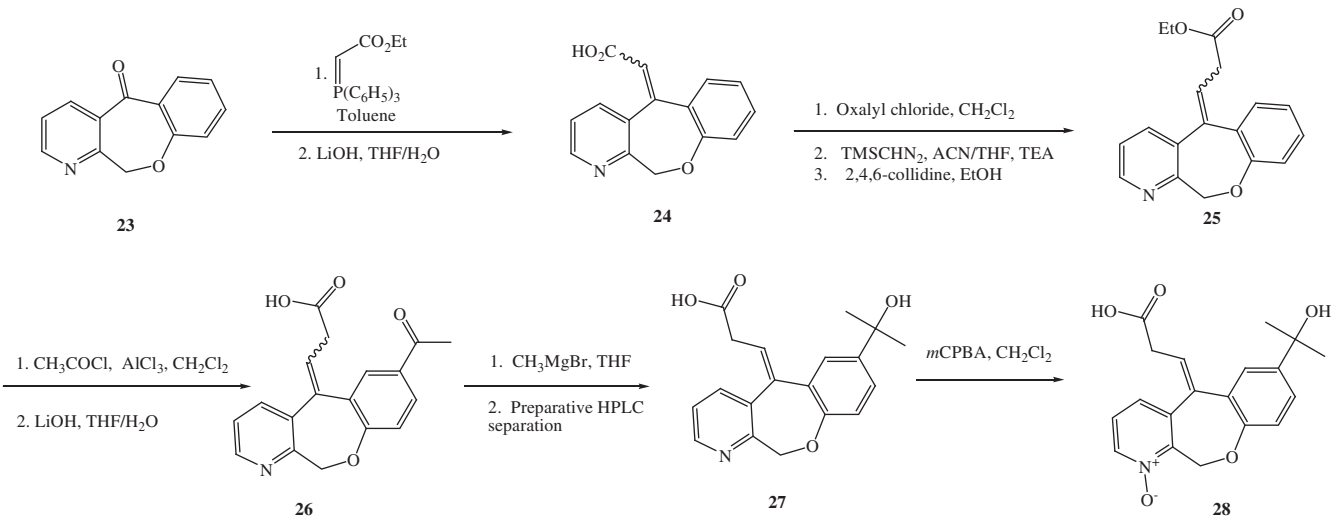
Metabolite M28 (22): For the preparation of M28 (as N-oxide of MLN3897) we chose selective oxidation of N-pyridine with selenium dioxide or hydrogen peroxide. Unfortunately in both cases we also formed significant impurities that were difficult to remove during chromatography. The total synthesis of target compound **22A** involved overnight treatment of 2-[(5*E*)-5-(3-bromopropylidene)-5,11-dihydro[1]benzoxepino[3,4-b]pyridin-7-yl]propan-2-ol (**20A**) with *m*-chloroperbenzoic acid (77%) in methylene chloride followed by alkylation with chiral piperidinol **12** as described in the last step for the preparation of tricyclic [^{14}C]-MLN3897 **(13)**.

M28 is a pharmacologically active metabolite and a labeled internal standard was required for monitoring and quantitation in clinical trials (Scheme 4). Commercially available [$^{13}C_2$]-acetyl chloride was used for the Friedel-Crafts acylation reaction of bromide **9**, followed by the Grignard reaction with $^{13}CD_3MgBr$ as previously described for the radiosynthesis of tricyclic-[^{14}C]-MLN3897. Treatment of **20B** with *m*-chloroperbenzoic acid (77%) in methylene chloride followed by alkylation with **12** afforded the internal standard of M28 (**22B**).



Scheme 4. Synthesis of M28 and its internal standard.

Metabolites M11 and M19 (27 and 28): Tricyclic ketone **23** was treated with carbethoxy methylene triphenyl phosphorane under Wittig conditions to form the ester of (2*E*/*Z*)-[1]benzoxepino[3,4-b]pyridin-5(11*H*)-ylideneacetic acid (**24**) in a ratio of 3:1. The ester was saponified to give carboxylic acid (**24**). Treatment of the acid chloride with trimethylsilyldiazomethane followed by the thermal treatment in the presence of ethanol and 2,4,6-collidine gave the ethyl ester of a homologated carboxylic acid (**25**)[10]. After saponification, the acid was subjected to Friedel-Crafts acylation and Grignard reagent as described earlier. The mixture of (*E*)- and (*Z*)-isomers of the carboxylic acid **27** was carefully separated by reverse phase chromatography to give M19. A small portion of the (*E*)-isomer was treated with *m*-chloroperbenzoic acid to form metabolite M11 (**28**) (Scheme 5).

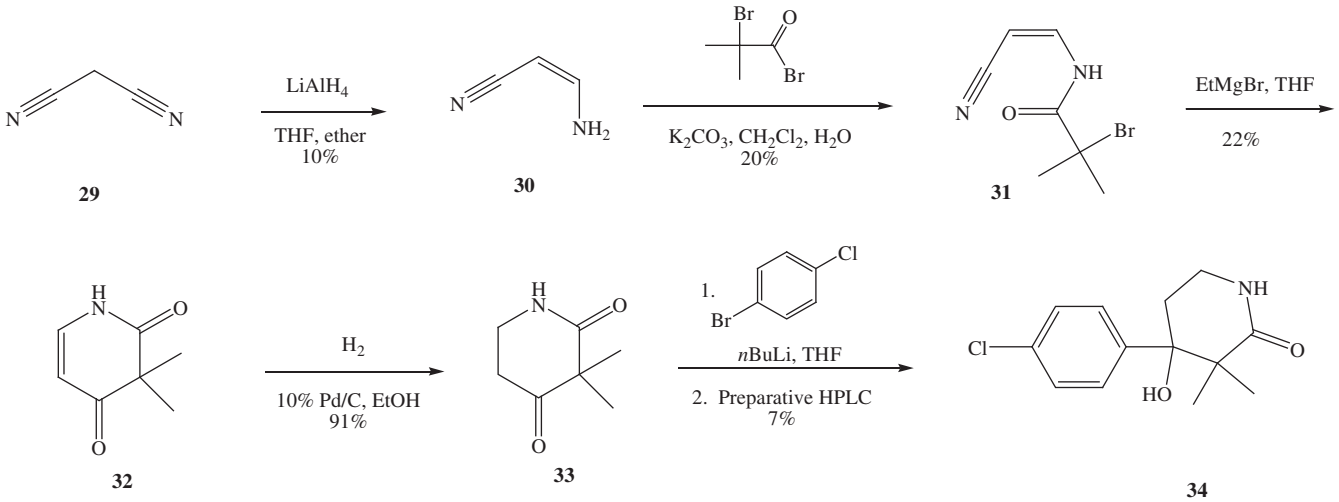


Scheme 5. Synthesis of M11 and M19.

Metabolite M44 (34): The structure of M44 was proposed to be a lactam resulting from *N*-dealkylation of MLN3897 and oxidation (Scheme 6). The synthesis of (*Z*)-*β*-aminoacrylonitrile (**30**) should be accessible from malonitrile (**29**) and LAH, but our attempts gave mixtures of (*Z*)- and (*E*)-**30** along with small amounts of 2-aminonicotinonitrile[11]. The separation of (*E*)- and (*Z*)-isomers was attempted by column chromatography, but the recovered product decomposed quickly when exposed to air at room temperature. A solution of 2-bromo-2-methylpropanoylbromide was added to a well-stirred 2-phase system composed of K_2CO_3 in water and **30** in CH_2Cl_2 to give (*Z*)-2-bromo-*N*-(2-cyanovinyl)-2-methylpropanamide (**31**). Treatment of **31** with a solution of ethylmagnesium

Journal of Labelled Compounds and Radiopharmaceuticals

bromide under reflux conditions followed by quenching in a mixture of ice and aqueous NH_4Cl provided 3,3-dimethylpyridine-2,4(1*H*,3*H*)-dione (**32**)[12]. Hydrogenation of the double bond followed by coupling of **33** with aryllithium gave the final product 4-(4-chlorophenyl)-4-hydroxy-3,3-dimethylpiperidin-2-one (**34**) which was isolated after reversed phase chromatographic purification.



Scheme 6. Synthesis of M44.

Conclusions: In support of ADME studies conducted in laboratory animals and humans we synthesized [^{14}C]-MLN3897 citrate by two separate routes allowing incorporation of a ^{14}C label in two different positions in the molecule. A stable isotope labeled version of MLN3897 was also prepared for use as an internal standard in bioanalytical assays.

In the preparation of C-14 labeled tricyclic MLN3897 using the method described by Carson *et al*., [1-^{14}C]-acetyl chloride was used as the starting material. The synthesis was completed in three radiochemical steps with an overall yield of 36% and radiochemical purity of 99%. Chlorophenyl [^{14}C]-MLN3897 was synthesized in one radiochemical step. Although the method was short, observed low yield was probably a result of introduction of the label at a sterically demanding site.

The predominant metabolites, M11, M19, M28 and M44 were synthesized with high chemical purity in order to provide positive identification.

Acknowledgements: The authors would like to thank the Inflammation Medicinal Chemistry and Process Chemistry groups at Millennium Pharmaceuticals for assistance with this project. We would also like to acknowledge the Radiochemistry group at Girindus America, Inc. for completing the GMP syntheses of [^{14}C]-MLN3897.

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THE SYNTHESES AND IN VITRO METABOLISM OF [^{14}C]APIXABAN, AN INHIBITOR OF BLOOD COAGULATION FXA

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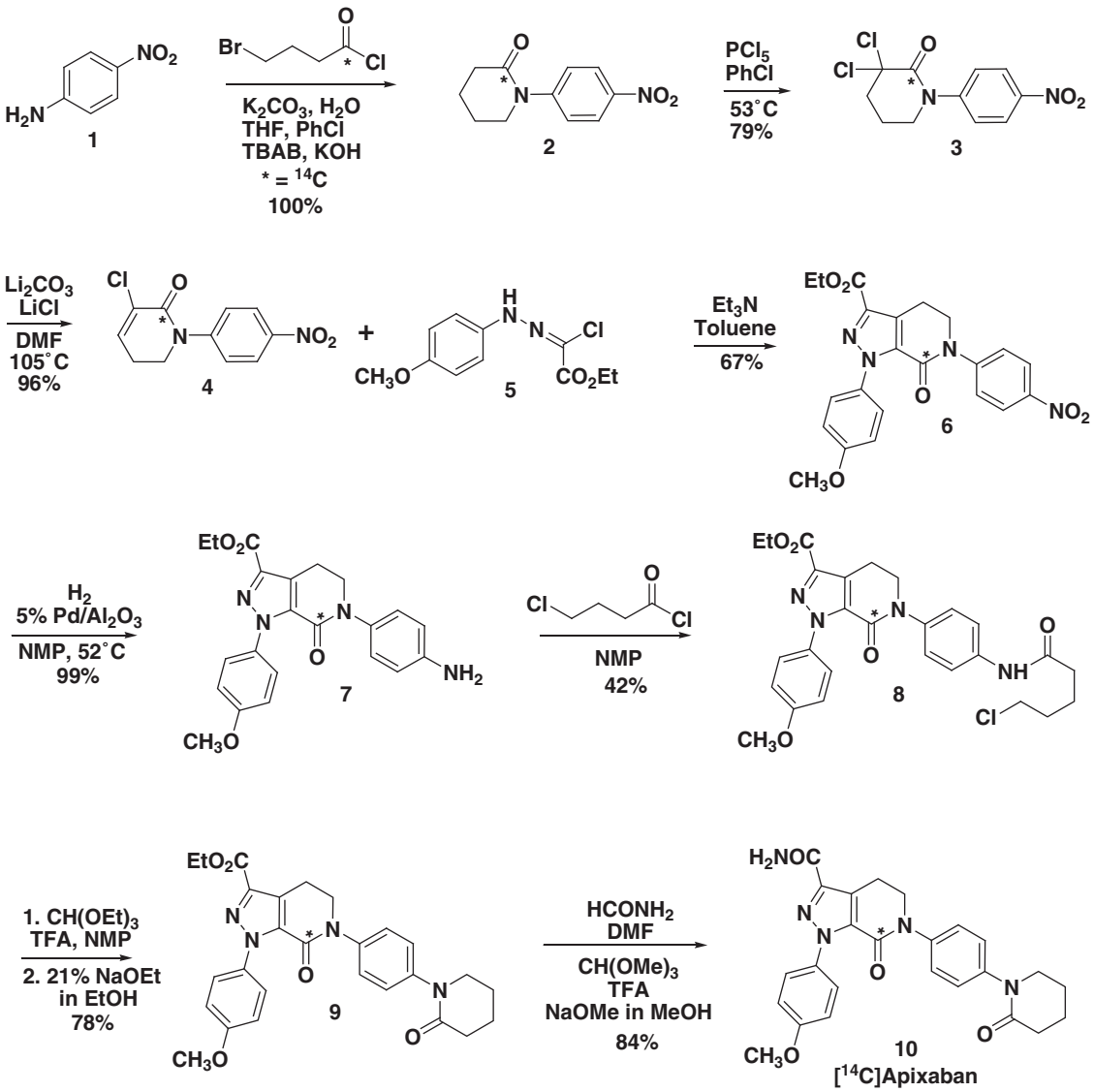
Abstract: Apixaban is a potent inhibitor of blood coagulation Factor Xa in the late stages of development. Two isotopologues of [^{14}C]Apixaban were synthesized for *in vitro* and *in vivo* metabolism studies. A nine step synthesis of [^{14}C]Apixaban, **10**, with the label in the central lactam ring was completed in 14% overall yield. A second synthesis of [^{14}C]Apixaban, **14**, with the C14 label in the outer lactam ring was completed in three steps in a 9% overall yield. No significant differences were observed between the metabolite profiles of **10** and **14,** [^{14}C]Apixaban, in both rat and human hepatocyte or microsomal incubations.

Keywords: Apixaban; Factor Xa; Thrombotic diseases; microsomes; hepatocytes; ADME

Introduction: Thrombotic diseases are the leading cause of death among people living in developed countries. Although the oral anticoagulant Warfarin is effective in both venous and arterial thrombosis, it suffers from a narrow therapeutic index, slow onset of therapeutic effects, several drug and dietary interactions, the need for monitoring and dose adjustments. Thus, the discovery and development of a highly potent, selective, efficacious and orally bioavailable inhibitor of blood coagulation Factor Xa as a replacement for Warfarin would be extremely valuable[1]. Apixaban represents such an agent and the preparation of [^{14}C] Apixaban was required for various preclinical and clinical studies. This paper describes the syntheses, purifications, analyses and characterizations of [^{14}C]Apixaban and its use in microsomal and hepatocyte *in vitro* metabolism studies.

Results and Discussion: Two isotopologues of [^{14}C]Apixaban, were synthesized. The first isotopologue, [^{14}C]**10,** synthesis is shown in Scheme 1. 4-Nitroaniline was reacted with [1-^{14}C]5-Bromovaleryl chloride under basic conditions to generate the nitro lactam **2** in quantitative yield. Product **2** was reacted with PCl_5 to produce the α,α- dichlorinated lactam, **3** in 79% yield. Product **3** was reacted with Li_2CO_3 at 105°C to produce the α,β-unsaturated lactam, **4** in 96% yield. Product **4** was reacted with Ethyl 2-chloro-2-(2-(4-methoxyphenyl)hydrazono)acetate **5** synthesized by the Bristol-Myers Squibb Process Chemistry Group to generate the pyrazole ring system product **6**, in 67% yield. The aromatic nitro group was reduced to aniline **7** with hydrogen gas and 5% Pd on Al_2O_3 in 99% yield.

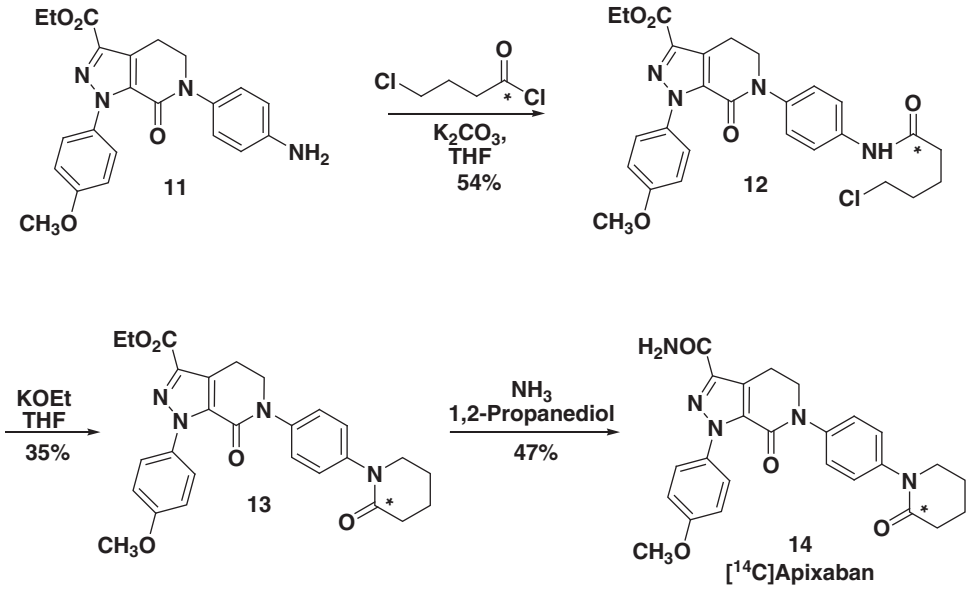


Scheme 1 – Synthesis of **10,** [^{14}C]Apixaban.

Journal of Labelled Compounds and Radiopharmaceuticals

The second lactam ring was prepared in three steps by reacting aniline product **7** with Chlorovaleryl chloride to generate amide **8** in 42% yield followed by a base promoted lactam ring formation in 78% yield. The final step involved the conversion of ethyl ester **9** to the primary amide with formamide to generate [^{14}C]Apixaban, **10**, in 84% yield with the Carbon-14 label located in the central lactam ring system. The overall yield for the nine step synthesis was 14%. The product was 99.27% radiochemically pure and 99.89% chemically pure. MS and ^{1}H-NMR matched those expected for [^{14}C]Apixaban. The Specific Activity was measured gravimetrically at 56.36 mCi/mmol or 122.1 μCi/mg.

A separate synthesis of [^{14}C]Apixaban with the carbon-14 label in the outer lactam ring system was completed and is shown in Scheme 2. In this synthesis, compound **7** was reacted with [1-^{14}C]Chlorovaleryl chloride to generate amide **11** in 54% yield. Product **11** was converted with Potassium ethoxide to the penultimate lactam **12** in 35% yield after purification by preparative HPLC. The penultimate ester was converted to the amide, [^{14}C]Apixaban, **13**, with ammonia in 47% yield. The three step sequence was completed in 9% overall yield. The final product was 100% Radiochemically pure. MS and ^{1}H-NMR matched those expected for the labeled product. The specific activity of [^{14}C]Apixaban, **13**, was measured at 49.46 mCi/mmol or 107.7 μCi/mg. The specific activity of a portion of the product was reduced to 2.49 mCi/mmol or 5.4 μCi/mg with unlabeled clinical grade Apixaban for use in the Human ADME Clinical Study[2].



Scheme 2 – Synthesis of **13,** [^{14}C]Apixaban.

The *in vitro* biotransformation profiles of both labels, **10** and **13**, in cryopreserved hepatocytes and liver microsomes from rats and humans were determined. At the completion of a 4–hr incubation, 83% and 92% of cells remained viable for rat and human hepatocytes, respectively. After 4-hr incubation, 44.1% and 51.0% of 7-Ethylcoumarin (EC) and 42.1% and 97.8% of 7-Hydroxycoumarin (HC) were metabolized by rat and human hepatocytes, respectively. In addition, significant amounts of 7-HC sulfate and 7-HC glucuronide were also observed. Thus, both hepatocyte preparations used in this study were enzymatically active for the 4-hr incubation. After 1-hr incubation, 71.2% and 90.2% of 7-EC was metabolized by rat and human liver microsomes, respectively. In addition, significant amount of 7-HC, a metabolite of 7-EC, was observed in 1-hr incubation samples. Thus, both microsomal preparations used in this study were enzymatically active for the 1-hr incubation.

The 1-, or 4-hr microsomal or hepatocyte incubation mixtures of [^{14}C]Apixaban (both **10** and **13**, at 1 and 10 μM), were extracted with ice-cold acetonitrile. The extraction recoveries of the total radioactivity from the incubation mixtures were quantitative for rat and human hepatocyte and microsomes for **13** and **10**. [^{14}C]Apixaban, both **10** and **13**, appeared to be relatively stable in hepatocyte incubation media and phosphate buffer at 10 μM. Greater than 97% of [^{14}C]Apixaban remained unchanged after a 1- or 4-hr incubation control. No detectable degradation products were observed in the 1 μM negative control incubations of both **13** and **10**.

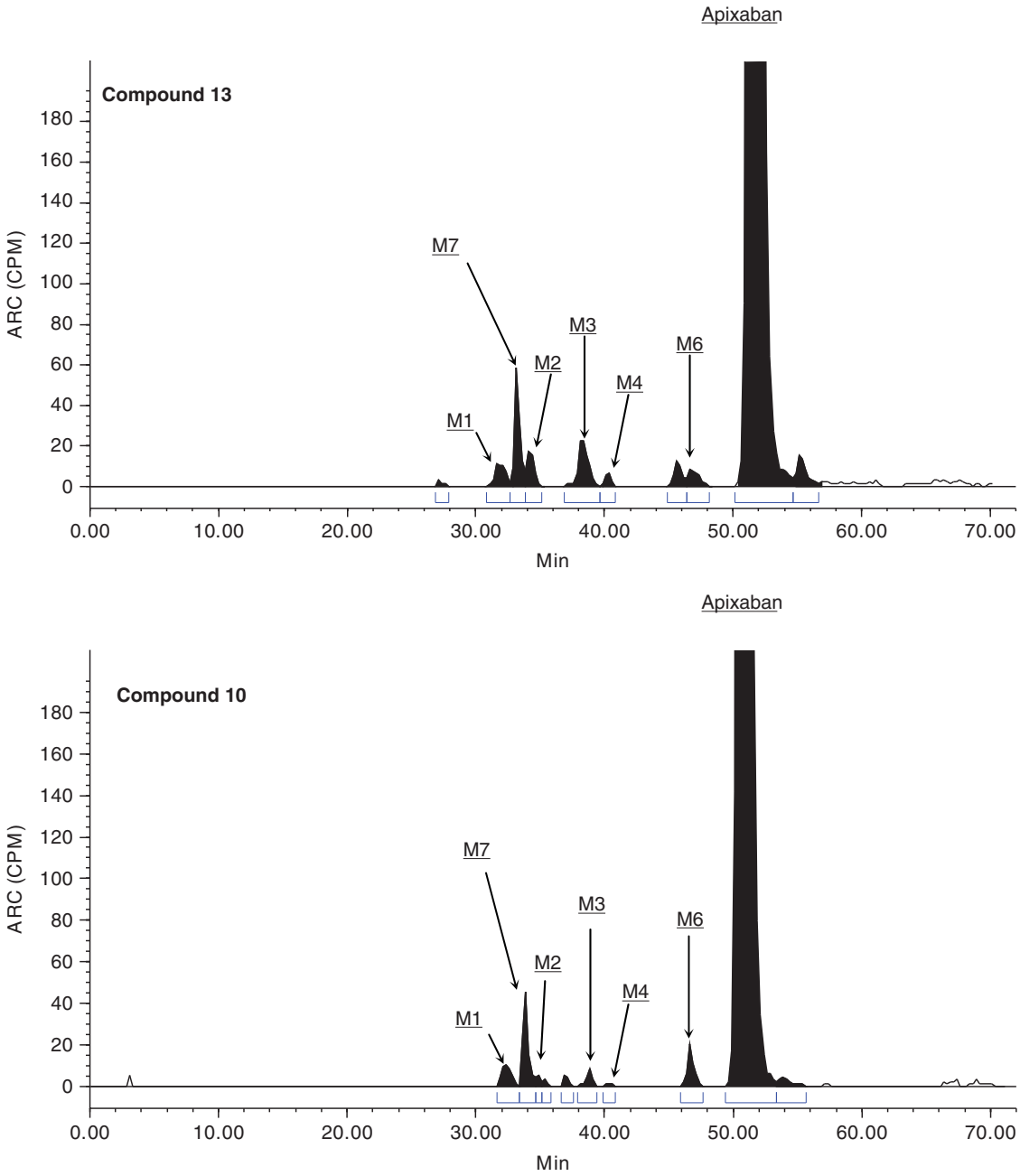


Figure 1. HPLC Radio-Chromatograms of 4–Hour Human Hepatocyte Incubations with 10 μM [^{14}C]Apixaban, Compounds **10** and **13**.

The metabolism of Apixaban in rat and human hepatocyte or microsomal incubations was very limited, with >92% and 95% of the total radioactivity being attributed to Apixaban after a 1- or 4-hr incubation. Six metabolites, M1-M4 and M7, were tentatively identified in hepatocytes and 5 metabolites, M2-M4 and M7 were tentatively identified in microsomal incubations. These *in vitro* metabolites were similar to the *in vivo* metabolites[2,3] M2 was identified as the O-desmethyl metabolite. M4 and M7 were hydroxylated metabolites. M1 was a sulfate conjugate of M2. M3 was a ring opened metabolite and M6 was the amide hydrolyzed metabolite. There were no significant differences observed between the metabolite profiles of both **10** and **13** of [^{14}C]Apixaban from both rat or human hepatocyte or microsomal incubations, see Figures 1 and 2. Metabolite profiles were similar for 1 and 10 μM incubations. Similar metabolic profiles of **10** and **13** supported the use of **13** for Human ADME studies and that experiments with compound **13** adequately assessed the metabolic profile of Apixaban.

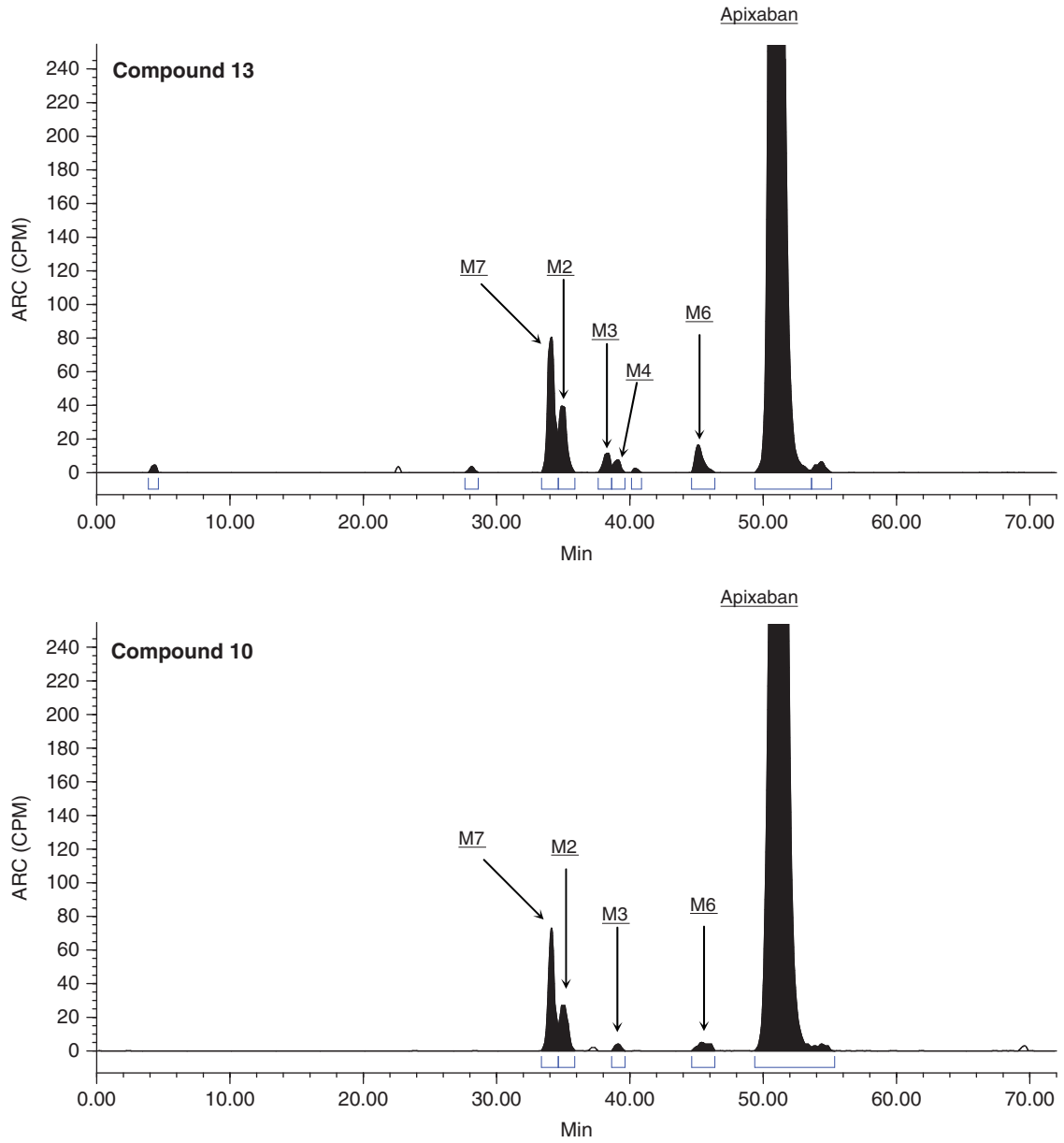


Figure 2. HPLC Radio-Chromatograms of 1–Hour Human Liver Microsomal Incubations with 10 μM [^{14}C]Apixaban, Compounds **10** and **13**.

Conclusion: Two separate syntheses of isotopologues of [^{14}C]Apixaban, **10** and **13**, were completed for various *in vitro* and *in vivo* studies. There were no significant differences observed between the metabolite profiles of **10** and **13** [^{14}C]Apixaban in both rat or human hepatocyte or microsomal incubations.

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