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Multiple strategies for the preparation of a sulfur-35 labeled NPC1L1 radioligand

Joseph P. Simeone [a,*], Matthew P. Braun [a], Joseph F. Leone [a], Peter Lin [b], Robert J. DeVita [b], Margarita Garcia-Calvo [c], Herb G. Bull [c], JeanMarie Lisnock [c], Dennis C. Dean [d]

[a] Department of Process Research, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065-0900, USA
[b] Department of Medicinal Chemistry, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065-0900, USA
[c] Department of Metabolic Disorders, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065-0900, USA
[d] Department of Drug Metabolism and Pharmacokinetics, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065-0900, USA

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ABSTRACT

During our effort to design a receptor binding assay to aid in the elucidation of the molecular mechanism of ezetimibe, we prepared a sulfur-35 containing radioligand which exhibits improved potency over the glucuronide conjugate of ezetimibe in both native enterocyte brush border membranes and membranes from cells expressing recombinant NPC1L1. Herein, we describe the different synthetic strategies which were used to obtain this compound as well as its effectiveness in the aforementioned assay.

Ezetimibe is an effective therapeutic for the reduction of low density lipoproteins and functions through the inhibition of cholesterol absorption from the small intestine into the bloodstream. The development of ezetimibe (**1**, Fig. 1) is uncommon in the modern drug discovery era in that its structure was optimized based on in vivo efficacy and active metabolite identification in the absence of an in vitro assay.[1] Almost immediately after oral administration in rodents, ezetimibe forms a glucuronide conjugate at the phenolic hydroxyl group.[2] This metabolite, ezetimibe glucuronide (**2**, Fig. 1) has been shown to have comparable potency to ezetimibe itself.[3] Furthermore, glucuronide formation in humans leads to enterohepatic circulation which localizes ezetimibe glucuronide at the site of action, the small intestine.[4]

The mechanism by which ezetimibe regulates cholesterol flux at the molecular level is becoming evident. Recent studies have resulted in an increased understanding of intestinal cholesterol absorption. The identification of Niemann-Pick C1 Like 1 Protein (NPC1L1)[5] was followed shortly thereafter by a study involving NPC1L1-deficient mice which demonstrated that NPC1L1 is involved in cholesterol uptake in enterocytes.[6] Evidence that this protein is the molecular target of ezetimibe was provided by the development of a binding assay and the finding that tritiated ezetimibe glucuronide binds to a single site in brush border membranes (BBMs) and does not bind to BBMs prepared from NPC1L1 knockouts.[7]

Since these findings, further studies aimed at probing the mechanism by which NPC1L1 is involved in cholesterol transport have been reported.[8] These include experiments utilizing cell lines[9] expressing recombinant and endogenous NPC1L1. Recently reported data provides evidence that NPC1L1 possesses binding sites for both ezetimibe and cholesterol and that ezetimibe binding may force a conformational change in NPC1L1 which prevents its interaction with cholesterol.[10] Furthermore, it has been shown that cholesterol binding to NPC1L1 causes an association with the clathrin/AP2 complex, resulting in internalization of both cholesterol and NPC1L1. Ezetimibe acts by inhibiting this process.[11]

Competitive binding studies often require the synthesis of ligands containing a high specific activity radiolabel. This label must be carefully chosen so as to ensure that the binding of the resulting radioligand is both specific and of high affinity. In cases where incorporation of tritium or iodine-125 does not meet these requirements, sulfur-35 may be used as a surrogate.[12] Sulfur-35 is most often introduced into a molecule in the form of a sulfonamide, via the treatment of an amine with methane[^{35}S]sulfonyl chloride.[13] While this method is both general and efficient, it can sometimes be problematic when the amine of interest suffers from poor solubility in methylene chloride. These cases often give rise to low conversions to [^{35}S]sulfonamides and large amounts of methane[^{35}S]sulfonic acid, arising from hydrolysis of the sulfene intermediate.[14] While the labeling reagent can sometimes be recycled, it is a time consuming process often resulting in low recovery.

During the course of our effort to elucidate the mode of action of ezetimibe we designed a sulfur-35 labeled radioligand (**3**,

* Corresponding author. Tel.: +1 732 594 4307; fax: +1 732 594 6921.
E-mail address: joe_simeone@merck.com (J.P. Simeone).

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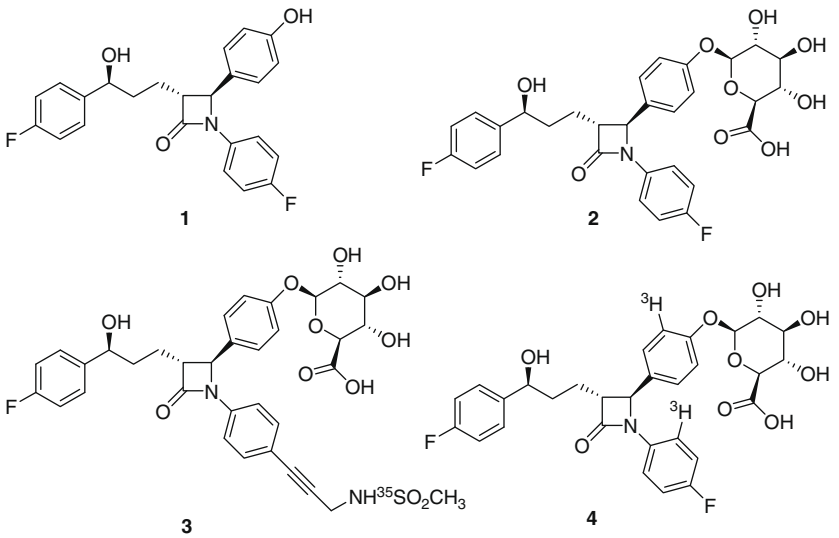


Figure 1. Structure of ezetimibe and its analogs.

Fig. 1) which exhibits increased binding affinity over ezetimibe glucuronide across species. However, the preparation of this compound was complicated by its lack of solubility in chlorinated solvents. To circumvent this problem, we have successfully employed two novel synthetic methods for the synthesis of sulfur-35 containing compounds. The first procedure involves a low mass palladium-mediated Sonogashira-type coupling reaction of an aryl iodide with a labeled propargylamine sulfonamide. Alternately, it can be efficiently prepared via incubation of its phenol precursor in the presence of dog liver microsomes.

Initial attempts to develop a binding assay to identify the target of ezetimibe focused on the use of native rat intestinal brush border membranes. Four radiotracers were prepared to be used as ligands in the assay. These included tritiated and I-125 containing ezetimibe and ezetimibe glucuronide. The ezetimibe analogs were quickly shown to be unstable in aqueous and slightly basic buffers. In addition, [^{125}I]ezetimibe was a very poor radioligand resulting in high background binding. Therefore, tritiated ezetimibe (**4**, Fig. 1) was initially used to characterize the binding across species.[7] When compound **3** was prepared as a precursor to a photoaffinity ligand, it was found to have increased potency in this assay. Furthermore, it was eventually shown to have a high affinity for recombinant NPC1L1 across species, including the mouse NPC1L1 receptor for which it is 27-fold more potent than tritiated ezetimibe glucuronide (Table 1).

The synthesis of unlabeled **3** has been previously reported.[15] The radiolabeled analog of this compound was initially prepared using standard chemistry (Scheme 1). In the presence of base, compound **5**[15] was treated with a dichloromethane solution of methane[^{35}S]sulfonyl chloride which was prepared from methane[^{35}S]sulfonic acid.[13] Hydrolysis of the methyl ester resulted in the desired radioligand. Unfortunately, this process proved to be unreliable, resulting in variable radiochemical yields due to the limited solubility of **5** in halogenated solvents. Several modifications of this route were attempted including the use of different bases and various protected analogs of **5**. However, these changes failed to result in a more reliable synthesis. In order to satisfy the need for additional quantities of this radioligand, the design of an alternate route was necessary.

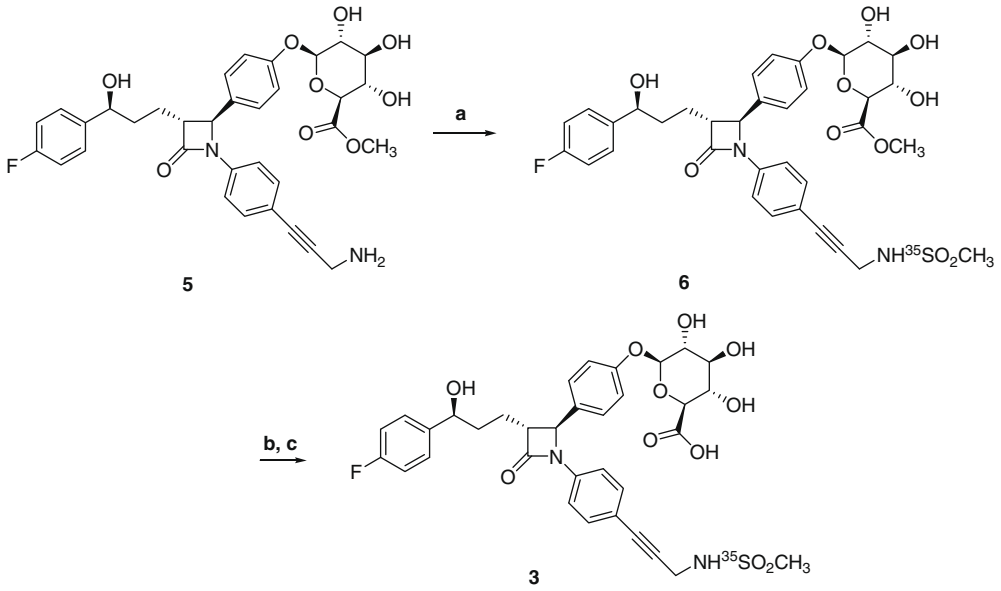
We envisioned that the molecule could be assembled via the Sonogashira cross-coupling[16] of labeled propargylamine sulfonamide and the appropriate iodoarene precursor (Scheme 2). Palladium-mediated chemistry using sulfur-35 labeled substrates was unknown at the time of this work.[17] We were uncertain as to whether the presence of palladium at concentrations many times in excess of a sulfur containing compound would be detrimental because of the affinity of sulfur for palladium compounds.[18] However, we were pleased to find that compound **8** could be successfully cross-coupled with iodoarene **9**[15] by utilizing the copper-free conditions developed by Leadbeater.[19] Standard protocol using tetrakistriphenylphosphine palladium (0) and copper(I) iodide resulted in a much longer reaction time. Sulfur-35 propargylamine sulfonamide **8** could be prepared from propargylamine and methane[^{35}S]sulfonyl chloride. Removal of the methyl ester followed by HPLC purification yielded the desired radioligand **3**. This process was used to successfully prepare several batches and consistently resulted in an average overall radiochemical yield of 20%. The specific activity ranged from 800 to 1100 Ci/mmol as determined by LC/MS.

While the Sonogashira chemistry proved to be a reliable route for the preparation of the radioligand, we found that the final HPLC purification was difficult because of the multicomponent reaction

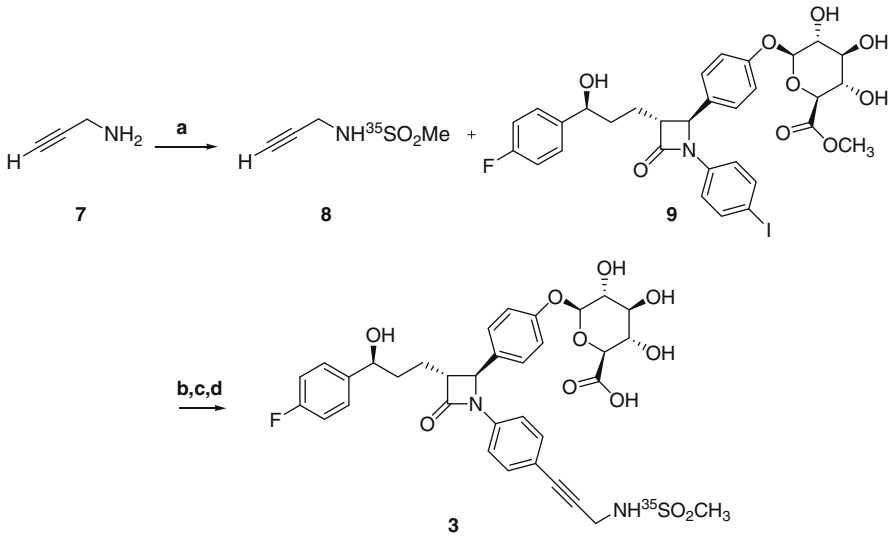
Table 1
NPC1L1 binding affinities across species expressed as IC_{50} (nM)

Compound	Human BBM	Rat BBM	Human NPC1L1	Rat NPC1L1	Mouse NPC1L1
3	4	4	4	4	44
4	150	127	200	173	1200

Mucosal intestinal membranes and membranes from human embryonic kidney (HEK) cells that have been transiently transfected with DNA constructs for expression of human, rat, or mouse NPC1L1 were prepared as described earlier.[7] Single-tube binding filtration assays were conducted in a final volume of 20 μL as reported previously using tritiated compound **4**.[7] Competition studies of ^{35}[S]-compound **3** binding by unlabeled compound **3** and ezetimibe glucuronide (12 concentrations) were conducted by using 1–5 nM ^{35}[S]-compound **3** and 5.7 μg/assay for human BBM, 0.3 μg for human NPC1L1, 1.5 μg for rat BBM, 1.3 μg for rat NPC1L1, and 15 μg for mouse NPC1L1 in 30 mM Hepes/117 mM NaCl/5.4 mM KCl, pH 7.4 containing 0.03% sodium taurocholate and 0.05% digitonin for maximal detection of binding sites in the samples. The values are the average of at least five independent experiments run in triplicates.



Scheme 1. Reagents and conditions: (a) methane[^{35}S]sulfonyl chloride, pyridine, CH_2Cl_2, room temperature, 15 min; (b) Et_3N, CH_3OH, H_2O, room temperature, 1 h; (c) HPLC purification.



Scheme 2. Reagents and conditions: (a) methane[^{35}S]sulfonyl chloride, pyridine, CH_2Cl_2, room temperature, 15 min; (b) $Pd(PPh_3)_2Cl_2$, Et_3N, DMF, 70 °C, 15 min; (c) Et_3N, CH_3OH, H_2O, room temperature, 1 h; (d) HPLC purification.

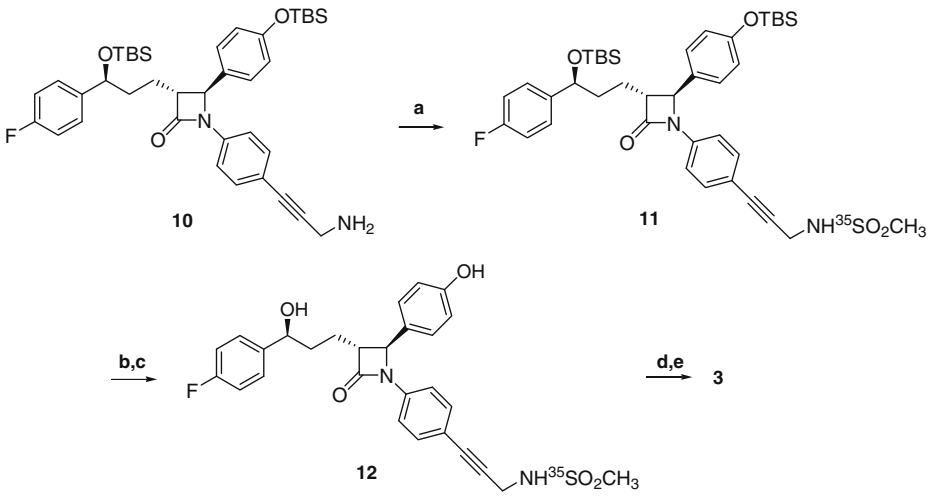
mixture. We therefore turned our attention to developing an alternate strategy which would allow us to prepare multi-millicurie amounts without the need for tedious purification. Installation of the glucuronide conjugate onto a radiolabeled precursor was considered as a way to circumvent this problem.

In addition to chemical methods, phenolic glucuronides can be prepared via enzymatic catalysis.[20] This method is limited to milligram amounts of the phenol substrate and is often low yielding. Because of this, enzymatic methods of preparation are most often utilized in metabolite identification studies. We reasoned that a sulfur-35 containing compound would be amenable to enzymatic catalysis because of the extremely low mass present.

A suitable glucuronidation precursor (Scheme 3) was available in the form of the diol protected propargyl amine derivative **10**.[21] This compound was converted to the [^{35}S]sulfonamide **11**. After *tert*-butyldimethylsilyl (TBS) deprotection, phenol **12** was incubated in the presence of dog liver microsomes with UDPGA as the glucoronyl donor and saccharic acid lactone as a β-glucuronidase inhibitor.

This process was repeated several times and each incubation proceeded smoothly to give radiochemical yields as high as 67%. It was, however, necessary to purify the phenol **12** by reverse phase HPLC before subjecting it to the incubation conditions or the yield was significantly lower. High specific activity tracers (699 to 1100 Ci/mmol) could be prepared in this manner. It was necessary to run multiple 2 mL incubations if the total radioactivity exceeded 5 mCi because repeated attempts to run 10 and 20 mCi incubations resulted in markedly slower reaction rates and low turnover. To avoid this problem, incubations were run in parallel and then combined for isolation and purification.

While the average overall radiochemical yield for this process was similar to the Sonogashira route (17%), the purification of the final compound was easier due to the clean conversion of the phenol to the glucuronide. Batches of the radioligand in excess of



Scheme 3. Reagents and conditions: (a) methane[^{35}S]sulfonyl chloride, pyridine, CH_2Cl_2, room temperature, 15 min; (b) 48% aq HF, CH_3CN, rt, 6 h; (c) HPLC purification (d) dog liver microsomes, bis-tris buffer pH 7.1, 0.1 M $MgCl_2$, 20 mM UDPGA, 0.1 M saccharic acid lactone, alamethicin, CH_3CN, water, 37 °C, 3 h; (e) HPLC purification.

10 mCi could be prepared and stored with minimal decomposition provided methanol was used as the storage solvent.

In conclusion, we have discovered a radioligand with high affinity for the NPC1L1 receptor across multiple species. This compound can be used as a diagnostic tool to further our understanding of intestinal cholesterol transport. Its preparation required us to expand the scope of sulfur-35 synthetic chemistry. In doing so, we have adapted both chemical as well as enzymatic reactions for the synthesis of a high specific activity radioligand. This work underscores the use of sulfur-35 as a viable alternative for iodine-125 and tritium.

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Supplementary data

Supplementary data (detailed experimental procedures for the preparation of compounds **3**, **6**, **8**, **11**, and **12**, radiochromatograms, and mass spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.051.

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