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Synthesis of Deuterium Labeled D_5-JPH203 and D_3-*N*-Acetyl JPH203

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ABSTRACT

JPH203 is a selective LAT1 inhibitor designed to significantly reduce essential uptake and induce apoptosis by the mitochondria-dependent intrinsic apoptotic signalling pathway. A phase II active metabolite of JPH203 is *N*-acetyl JPH203. Herein, we report the synthesis and characterization of deuterium labelled d_5-JPH203 and d_3-*N*-acetyl JPH203. JPH203 is currently in Phase II clinical trials. These compounds have been successfully used as internal standards for liquid chromatography mass spectrometry-mass spectrometry (LC/MS-MS) from biological samples. Comprising fifteen steps, the synthesis of d_5-JPH203 utilizes d_5-benzoyl chloride. Whereas d_3-acetyl JPH203 uses d_3-acetylchloride and prepared in eighteen synthetic steps.

Keywords: d_5-JPH203; d_3-N-Acetyl-JPH203; LAT1 (L-Amino Acid Transporter 1); Internal Standards

Abbreviations: MTOR: Mammalian Target of Rapamycin; LAT: L-Amino Acid Transporter; SAR: A Structure-Activity Relationship; IS: Internal Standards; PPSE: Polyphosphate

Introduction

The L-amino acid transporter 1 (LAT1) is an antiporter that catalyzes the cross-membrane flux of large amino acids with neutral side chains such as the L-isomers phenylalanine, tryptophan, leucine, and tyrosine in a sodium- and pH- independent manner [1]. The structure of LAT1 consists of 12 transmembrane helices that are arranged in a 5+5 two-fold inverted repeats [2]. It forms a heterodimer with the heavy chain 4F2hc cell-surface antigen via a disulfide bridge between C164 of LAT1 and C210 of 4F2hc [2]. This heavy chain is a type II membrane glycoprotein that plays a large role in the stability of LAT1, its localization to the outer lipid bilayer, and in the transport activity of the complex [1]. LAT1 is highly expressed in the blood-brain barrier, placenta, immune T cells, and numerous types of cancer cells [2]. In cancer cells specifically, LAT1 is upregulated to support large-scale growth and proliferation [3]. One study of bladder carcinoma provides an example. Leucine is an essential amino acid that is taken into the cell by LAT1 and activates mammalian target of rapamycin (mTOR) signaling pathway that serves as central regulator of cell metabolism, growth, proliferation, and survival. Increased LAT1 expression increases L-leucine uptake. This then triggers mTOR signaling to further contribute to bladder carcinoma progression and treatment resistance [3]. In this same study, JPH203 was used as the therapeutic agent with LAT1 as the target.

A structure-activity relationship (SAR) study has recently been described, and a summary of the Phase I clinical trial has also recently published [4,5]. JPH203 is an LAT1-selective *compound* that functions as a competitive inhibitor against other substrates when interacting with the transporter [6]. It induces blocking of LAT1-depedent large amino acids to disrupt amino acid homeostasis, mTOR activity, and thus proliferation of cancer cells [7]. JPH203 also

aids in mitochondria-dependent apoptosis, as previously seen in Saos2 human osteosarcoma cells [8]. JPH203 activates apoptosis by upregulating pro-apoptotic factors such as Bad, Bax, and Bak along with the active form of caspase [9]; downregulation of anti-apoptotic factors like Bcl-2 and Bcl-xL was also observed [8] It has been previously shown that the major route of biotransformation is phase II metabolism to produce *N*-acetyl JPH203 via *N*-acetyltransferase [9]. During some pre-clinical work, tamoxifen was used as the internal standard [9]. An internal standard is expected to be stable, have similar physical properties as the target molecule, and elutes from the HPLC in close proximity to the peak of the target molecule. The ideal molecules would then be a deuterated form of JPH203 and its metabolite NAc-JPH203, and these compounds are essential as the results of Phase I study has been summarized and Phase II clinical trial is underway in Japan [5] This paper discusses the synthesis of internal standards d_5-JPH203 and d_3-NAc JPH203.

Materials and Methods

All chemicals utilized for synthesis were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO), unless otherwise stated. The 3-nitro-salicylic acid starting material was purchased from Tokyo Chemical Industry, Co. All reactions were carried out under a nitrogen atmosphere unless otherwise noted. The 1H and ^{13}C NMR spectra were obtained on a Bruker 400 MHz NMR. Solvents $CDCl_3$ and DMSO-d_6, MeODwere used for NMR analysis and are specified with each synthesized compound. Analytical thin-layer chromatography was performed on silica gel plates (Baker-flex Silica Gel IB2-F) using a short wavelength for UV activity detection (Analytik Jena US, 6 Watt, 254/365nm, 0.12 Amps). Standard grade silica (40-63 µm) was packed manually for column chromatography purification.

Synthetic Detail for d_5-JPH203

Benzoyl Chloride-d_5 (1): Benzoic acid-d_5 (8.00 g, 58.3 mmol) was suspended in dry toluene (100 mL) in a flask to which thionyl chloride (29.6 mL) was added dropwise. The temperature was increased to 110°C and allowed to stir overnight. Toluene was then evaporated under vacuum. The product was thrice treated with dry toluene (10 mL each time). A plum-colored oil was obtained. Yield: 8.70 g (96%). The product was used to continue the synthesis without further purification.

d_5-Methyl 3-Benzamido-2-Hydroxybenzoate (3): 3-Amino methyl salicylate (2) (7.78 g, 53.4 mmol) was suspended in dry THF (90 mL) and cooled to 5°C. To this, *N*,*N*-dimethylaniline (11.8 mL) was added dropwise and stirring ensued for 15 min. Benzoyl chloride-d_5 (1) (8.7 g) was dissolved in dry THF (10 mL) and added dropwise to the reaction mixture. The reaction mixture was stirred for an hour before 10 mL of water was added to the flask. The product was extracted into 70 mL ethyl acetate layer and washed with 10% HCl and brine. The organic layer was dried with anhydrous sodium sulfate, filtered and the remaining solvent evaporated under pressure to give an orange-yellow solid. Yield: 8.60 g (67%). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 3.95 (3H, s, CH_3), 6.99-7.03 (1H, m, CH aromatic), 7.67-7.70 (1H, m, CH aromatic), 7.95-7.96 (1H, m, CH aromatic), 9.67 (1H, s, NH), 10.92 (1H, s, OH aromatic). ^{13}C- NMR (100 MHz, $CDCl_3$): δC 52.5, 111.7, 119.3, 123.9, 125.1, 126.2, 127.6, 128.5, 131.5 134.6, 150.7, 165.3, 170.9 ppm.

d_5-Methyl 3-Benzamido-2-Hydroxy-5-Nitrobenzoate (4): d_5-Methyl 3-benzamido-2-hydroxybenzoate (3) (8.60 g, 31.1 mmol) was dissolved in acetic acid (120 mL) and the mixture was cooled to 15 °C. A solution of 69% nitric acid (6.9 mL) was added dropwise for 10 min and followed by stirring the reaction mixture at room temperature for 1.0 hr. Afterwards, 20 mL of ice water was added and stirring ensued for another 10 minutes. The precipitate was filtered by vacuum, washing with water and diethyl ether. The product continued to dry under high vacuum overnight to obtain a light tan solid. Yield: 6.50 g (65%). [1] H-NMR (400 MHz, DMSO-d_6): δH 3.98 (3H, s, CH_3), 8.45 (1H, s, CH aromatic), 8.92 (1H, s, CH aromatic), 9.98 (1H, s, OH aromatic) ppm.

d_5-Methyl 5-Nitro-2-Phenyl-1,3- Benzoxazole-7- Carboxylate (5): d_5-Methyl 3-benzamido-2- =hydroxy-5-nitrobenzoate (4) (6.50 g, 20.2 mmol) was combined with trimethylsilyl polyphosphate and 1,2-dichlorobenzene in a reaction flask. This mixture was then heated to 150°C and stirred for 5.0 hr. Upon completion, the reaction mixture was poured onto ice water. The precipitate was filtered by vacuum, washing with three 25 mL batches of n-hexanes, then ice-cold methanol. The solid was collected and dried under high vacuum. A colorless solid was obtained. Yield: 6.4 g (80%). ^{1}H-NMR (400 MHz, $CDCl_3$): δH 4.14 (3H, s, CH_3), 8.82 (1H, s, CH aromatic), 8.94 (1H, s, CH aromatic). ^{13}C-NMR (100 MHz, $CDCl_3$): δC 53.01, 115.06, 119.67, 122.64, 125.28, 127.69, 128.52, 132.53, 144.23, 144.89, 152.99, 162.72, 166.85 ppm.

d_5-5-Nitro-2-Phenylbenzo[*d*]Oxazole-7-Carboxylic Acid (6): d_5-Methyl 5-nitro-2- phenylbenzo[*d*]oxazole-7-carboxylate (5) (6.40 g, 21.1 mmol) was suspended in equal amounts of THF, MeOH, and water (40 mL each). To this, lithium hydroxide monohydrate (1.77 g) was added, and the reaction stirred overnight at room temperature. Ethyl acetate was added for dilute the reaction mixture and a pH of 4.0 was achieved by adding 1.0 M HCl dropwise with stirring. The precipitate was then filtered by vacuum and washed with water to give a mustard-yellow solid. Yield: 7.40 g (80%). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 8.68 (1H, s, CH aromatic), 8.92 (1H, s, CH aromatic), 14.14 (1H, s, COOH). ^{13}C-NMR (100 MHz, DMSO-d_6): δC 115.60, 122.52, 126.08, 126.95, 128.50, 129.23, 133.20, 143.25, 144.68, 153.17, 164.78, 165.90 ppm.

d_5-(5-Nitro-2-Phenylbenzo[*d*]Oxazol-7-yl)Methanol(7): d_5-5-Nitro-2-phenylbenzo[*d*]oxazole-7-carboxylic acid (6) (5.40 g, 18.7 mmol) was suspended in THF (81 mL). After triethylamine (3.12mL,

22.4mmol) was added, the reaction was stirred at room temperature for 30 min followed by cooling the reaction mixture to -10°C. To this, ethyl chloro-carbonate (2.13 mL, 22.4 mmol) was added dropwise, and the reaction mixture was stirred for another 30 min. Next, sodium borohydride (1.65 g, 43.6mmol) was added and stirred for another 30 min, THF/water (68 mL/14 mL) was added dropwise over a span of 3 hr, simultaneously bringing the temperature from -10°C to -6°C. The reaction liquid was poured into a beaker of ice water and the precipitated solid was filtered while washing with water. The resulting precipitate was suspended in THF/MeOH (75 mL/75 mL) with a dropwise addition of 1.0 M NaOH (14.6mL). The mixture was stirred at room temperature for 4.5 hr before the reaction mixture was poured onto ice water. The precipitate was filtered by vacuum filtration with water washed to give a peach-colored solid. Yield: 1.10 g (22%). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 4.93 (2H, s, CH_2), 5.84 (1H, s, OH), 8.30 (1H, s, CH aromatic), 8.48-8.49 (1H, m, CH aromatic). ^{13}C-NMR (100 MHz, DMSO-d_6): δC 57.6, 114.4, 119.2, 125.7, 127.6, 127.8, 128.5, 132.5 142.0, 145.5, 151.6, 165.4.

d_5-(5-Nitro-2-Phenylbenzo[*d*]Oxazol-7-Yl) Methyl Methanesulfonate (8): d_5-(5-Nitro-2-phenylbenzo[d]oxazol-7-yl) methanol (7) (1.00 g, 3.63 mmol) was dissolved in dry THF (25 mL) and the flask containing the reaction mixture was submerged in an ice bath. With stirring, triethylamine (1.52 mL) was added, followed by methyl-sulfonyl chloride (422 µL, 5.45 mmol) in a dropwise fashion. The mixture was stirred on ice for 30 min before bringing the reaction to room temperature to stir for another 3 hr. The reactant liquid was poured onto ice to precipitate the product, which was isolated by vacuum filtration. The residue was washed with water and di-isopropyl ether to give a colorless solid. Yield: 1.00 g (78%). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 3.41 (3H, s, CH_3), 5.75 (2H, s, CH_2), 8.48 (1H, s, CH aromatic), 8.71 (1H, s, CH aromatic) ppm.

d_5-Methyl 2-((Tert-Butoxycarbonyl) Amino)-3-(3,5-Dichloro-4-((5-Nitro-2-Phenyl- Benzo[*d*]Oxazol-7-Yl) Methoxy) Phenyl) Propanoate (10): d_5-(5-Nitro-2-phenylbenzo[*d*]oxazol-7-yl) methyl methanesulfonate (8) (948 mg, 2.68 mmol) was dissolved in THF (27 mL) and acetone (27 mL). To this mixture, methyl 2-(tert-butoxy carbonyl) amino)-3-(3,5-dichloro-4-hydroxyphenyl) propanoate (9) (1.18 g, 3.24 mmol), sodium iodide (405 mg), and potassium carbonate (495 mg) were added and stirred at room temperature for 21 hr. The reaction mixture was filtered out over vacuum and the filtrate was concentrated to half of its original volume. The resulting organic layer was washed with water, 5% aqueous sodium thiosulfate, and brine. The organic layer was then dried with sodium sulfate, filtered and evaporated under vacuum to give a colorless solid. Yield: 1.60 g (96%). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 1.32 (9H, s, 3 x CH_3), 2.76-2.82 (1H, m, CH_2), 2.99-3.04 (1H, m, CH_2), 3.64 (3H, s, CH_3), 4.21- 4.27 (1H, m, CH), 5.50 (2H, s, CH_2), 7.32-7.34 (1H, m, NH), 7.44 (2H, s, CH aromatic), 8.52-8.53 (1H, d, J 4 Hz, CH aromatic), 8.73-8.74 (1H, d, J 4 Hz, CH aromatic). ^{13}C-NMR (100 MHz, DMSO-d_6): δC 28., 35.6, 52.4, 54.8, 55.4, 69.1, 78.9, 116.4, 121.2, 121.8, 125.5, 128.4, 130.5, 137.3, 142.5, 145.4, 148.9, 152.9, 155.8, 165.6, 172.5.

d_5-Methyl 3-(4-((5-Amino-2-Phenylbenzo[*d*] Oxazol-7-Yl) Methoxy)-3,5-Dichlorophenyl)-2- ((Tert-Butoxycarbonyl) Amino) Propanoate (11): Compound 10 (1.24 g, 1.99 mmol) was dissolved in THF (29 mL) and isopropanol (29 mL) and subsequently heated to and internal temperature of 60°C. To this, 5.0 M ammonium chloride (6.38 mL) and iron powder (2.23 g) were added and stirred for 2 hr. The reactant liquid was filtered on celite, and the filtrate was concentrated to half of its original volume. Hot ethyl acetate was used to wash the impurities on the celite; the resulting filtrate from these washes was combined with the reduced filtrate. The resulting organic layer was washed with water and brine, followed by drying with sodium sulfate. Filtration and evaporation of solvent gave a brown-yellow solid. Yield: 480 mg (41%). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 1.32 (9H, s, CH_3), 2.77-2.83 (1H, m, CH_2), 3.00-3.05 (1H, m, CH_2), 3.64 (3H, s, CH_3), 4.22- 4.28 (1H, m, CH), 5.22 (2H, s, CH_2), 6.84-6.85 (1H, m, CH aromatic), 6.89- 6.90 (1H, m, CH aromatic), 7.43 (2H, s, CH aromatic). ^{13}C-NMR (100 MHz, DMSO-d_6): δC 28.5, 30.9, 35.6, 49.1, 52.4, 54.8, 70.1, 78.9, 103.4, 114.4, 119.6, 127.1, 128.6, 130.5, 136.9, 141.7, 143.2, 147.1, 149.1, 155.8, 162.4, 172.5 ppm.

d_5-3-(4-((5-Amino-2-Phenylbenzo[*d*]Oxazol-7-yl) Methoxy)-3,5-Dichlorophenyl)-2-((Tert-Butoxycarbonyl) Amino) Propanoic Acid (12): d_5-Methyl 3-(4-((5-amino-2-phenyl-benzo[d] oxazol-7-yl) methoxy)-3,5-dichlorophenyl)-2-((tert- butoxycarbonyl) amino)propanoate (11) (258 mg, 0.436 mmol) was combined with THF (5.0 mL) and the reaction flask was cooled over ice. Drops of 0.5 M lithium hydroxide aqueous solution (1.31 mL) were added and stirring continued on ice for an additional hour. Afterwards, the reaction liquid was concentrated under pressure to half of its original volume. Two milliliters of deionized water was added, and the pH was decreased to 4.0 upon addition of 10% aqueous citric acid solution. The product was extracted into a layer of ethyl acetate-THF (10:1) which was washed with brine. The organic layer was dried over sodium sulfate, filtered and the remaining solvent was evaporated under pressure to give a pale-yellow solid. Yield: 173 mg (72%). Intermediate 12 was used as such for the next step.

d_5-JPH203 (13): d_5-3-(4-(5-Amino-2-phenyl benzo[d]oxazol-7-yl)methoxy)-3,5-dichloro phenyl)-2-(tert-butoxycarbonyl) amino) propanoic acid (12, 173 mg, 0.299 mmol) was suspended in THF (1.5 mL) and the reaction mixture was cooled on ice with stirring. 4.0 M HCl-dioxane (2.3 mL) was added dropwise and the reaction mixture was stirred on ice for 1 hr. The reaction mixture was then brought to room temperature and stirred for 2 days. Isopropyl ether was then added to the reaction mixture to precipitate the product. The product was isolated by vacuum filtration to yield a light tan solid. Yield: 80

%. ^{1}H-NMR (400 MHz, CD3OD): δH 3.09-3.17 (1H, m, CH_2), 3.22-3.33 (1H, m, CH_2), 4.26-4.30 (1H, m, CH), 5.49 (2H, bs, CH_2), 7.43 (2H, s, CH aromatic), 7.57 (1H, s, CH aromatic), 7.68 (1H, s, CH aromatic). ^{13}C-NMR (100 MHz, CD3OD): δC 34.72, 53.38, 68.52, 112.36, 119.27, 121.33, 125.84, 127.03, 128.42, 129.60, 130.05, 131.27, 133.28, 142.80, 147.43, 150.05, 164.83, 169.51 ppm.

Synthetic Detail for d_3-*N*-Acetyl JPH203 (20)

d3-Methyl 3-(4-((5-Acetamido-2-Phenyl Benzo[d]Oxazol-7-yl) Methoxy)-3,5-Dichlorophenyl)- 2-((Tert-Butoxycarbonyl) Amino) Propanoate (17): Methyl 3-(4-((5-amino-2- phenylbenzo[d] oxazol-7-yl)methoxy)-3,5-dichlorophenyl)-2-((tert-butoxycarbonyl) amino) propanoate (16, 506 mg, 0.863 mmol) was dissolved in pyridine (12 mL). To this, d3-acetyl chloride (73.7 µL, 0.95mmol) was added dropwise. The mixture stirred at room temperature overnight. The following morning, pyridine was evaporated under pressure and ethyl acetate (10 mL) was added to the residue. The precipitate was filtered out over vacuum, and the filtrate was washed with brine. The organic layer was dried with anhydrous sodium sulfate, filtered, and the solvent was evaporated to give a crude compound. The compound was subjected to column chromatography, and the desired product eluted at 2.5% MeOH/ DCM to give a colorless solid. Yield: 358 mg (67%). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 1.33 (9H, s, 3 x CH_3), 2.76-2.83 (1H, m, CH_2), 2.99-3.04 (1H, m, CH^2), 3.65 (3H, s, CH_3), 4.25- 4.26 (1H, m, CH), 5.31 (2H, s, CH_2), 7.33 7.35 (1H, d, J 8 Hz, NH), 7.44 (2H, s, CH aromatic), 7.63-7.70 (4H, m, CH aromatic), 8.15-8.19 (3H, m, CH aromatic), 10.20 (1H, s, NH). ^{13}C-NMR (100 MHz, DMSO-d_6): δC 28.22, 28.51, 35.62, 52.40, 54.78, 69.75, 78.87, 110.51, 118.40, 119.74, 126.74, 127.64, 128.53, 129.81, 130.52, 132.51, 137.04, 137.06, 142.29, 145.18, 149.03, 155.81, 163.29, 168.92, 172.50 ppm.

d_3-3-(4-((5-Acetamido-2-Phenylbenzo[d] Oxazol-7-Yl) Methoxy)-3,5-Dichlorophenyl)-2-((Tert- Butoxycarbonyl) Amino) Propanoic Acid (18): d_3-Methyl 3-(4-((5-acetamido-2-phenyl benzo[d]oxazol-7-yl) methoxy)-3,5-dichloro phenyl)-2-((tert-butoxycarbonyl) amino) propanoate (17) (288 mg, 0.458 mmol) was dissolved in THF (7.0 mL) and stirred over ice. To this, 0.5 M aqueous lithium hydroxide (1.39 mL) was added dropwise. The reaction was stirred over ice for an hr, then brought to room temperature to stir overnight. Upon completion, the reaction mixture was concentrated to half of its original volume. To this, H2O (2.0 mL) was added, and the pH was dropped to 4 by adding 10% aqueous citric acid solution (1.2 mL). The precipitate was filtered over vacuum to give a white solid. Yield: 270 mg. Intermediate 18 was used as such for the next step.

d_3-N-Acetyl JPH203 in TFA salt (19): d_3-3-(4-((5-Acetamido-2-phenylbenzo[d]oxazol-7-yl) methoxy)-3,5-dichlorophenyl)-2-((tert-butoxy carbonyl)amino)propanoic acid (18) (145 mg, 0.235 mmol) was suspended in dry DCM (5 mL) in a flask to which trifluoroacetic acid (1.0 mL) was added dropwise. The reaction mixture stirred at room temperature overnight. Afterwards, the solvent and TFA were evaporated under pressure and the residue was washed thrice with isopropyl ether to give a tan solid. Compound 19 was used as such to make its HCl salt.

d_3-*N*-Acetyl JPH203 as HCl Salt (20): d_3-*N*-Acetyl JPH203 in TFA salt (19) (285 mg, 0.464 mmol) was dissolved in 1,4-dioxane (2.0 mL) in a flask to which 4.0 M HCl-1,4-dioxane (1.0 mL) was added dropwise. The reaction stirred for 5 min at room temperature. Upon completion, the solvent was evaporated under vacuum and the residue was washed thrice with 5.0 mL isopropyl ether to give a light tan solid. Yield: 253 mg (quant.). ^{1}H-NMR (400 MHz, DMSO-d_6): δH 3.08- 3.10 (1H, m, CH_2), 3.15-3.17 (1H, m, CH_2), 4.25-4.28 (1H, m, CH), 5.34 (2H, s, CH_2), 7.49 (2H, s, CH aromatic), 7.64-7.65 (3H, t, J 2 Hz, CH aromatic), 7.740-7.743 (1H, d, J 1.2 Hz, CH aromatic), 8.15-8.17 (3H, m, CH aromatic), 8.41 (2H, s, NH2), 10.29 (1H, s, NH). ^{13}C-NMR (100 MHz, DMSO-d_6): δC 29.48, 34.71, 53.12, 69.79, 110.59, 118.61, 119.61, 126.72, 127.63, 129.0, 129.84, 130.93, 132.53, 134.33, 137.06, 142.27, 145.26, 149.65, 163.28, 169.0, 170.57 ppm.

Results and Discussion

The structure and synthesis of d_5-JPH203 and d_3-*N*-acetyl JPH203 are illustrated within the experimental section as (Scheme 1 & Scheme 2), respectively. The methods for synthesizing both compounds were based off the patents for JPH203 synthesis. However, some steps were modified in order to achieve higher yields that required limited purification steps. Preparation of intermediates (2), (9), and (14) can be found in the supplementary materials section.

Preparation of d_5-JPH203

The deuterated benzoyl chloride (1) was reacted with the amine (2) in presence of base to afford deuterated amide (3) in 67% yield after recrystallization. Regioselective nitration at the 5th position afforded (4) after (3) was reacted with a dropwise addition of 69% nitric acid in water. Cyclization of (4) yielded best results with a 50/50 mixture of 1,2-dichlorobenzene and trimethylsilyl polyphosphate (PPSE). Cyclized intermediate (5) was obtained by vacuum filtration of its water-insoluble precipitate. Synthesis of (6) originally required addition of 1.0 M NaOH to a suspension of the starting material in methanol, however starting material persisted after the reaction ran overnight. Increasing the molarity of the NaOH aqueous solution resulted in significant loss of compound, perhaps due to degradation of the staring material in the presence of a strong base. Therefore, substituting the base with lithium hydroxide was preferred for synthesis of both d_5-JPH203 and d_3-*N*-acetyl JPH203. Carboxylic acid reduction was achieved by first forming a non-isolated anhydride intermediate and then reducing with sodium borohydride to give a yield of 22%. As addressed in the original patent, the reduction did not proceed with LiAlH4, therefore sodium borohydride was used on an anhydride intermediate to give the alcohol (7). The alcohol

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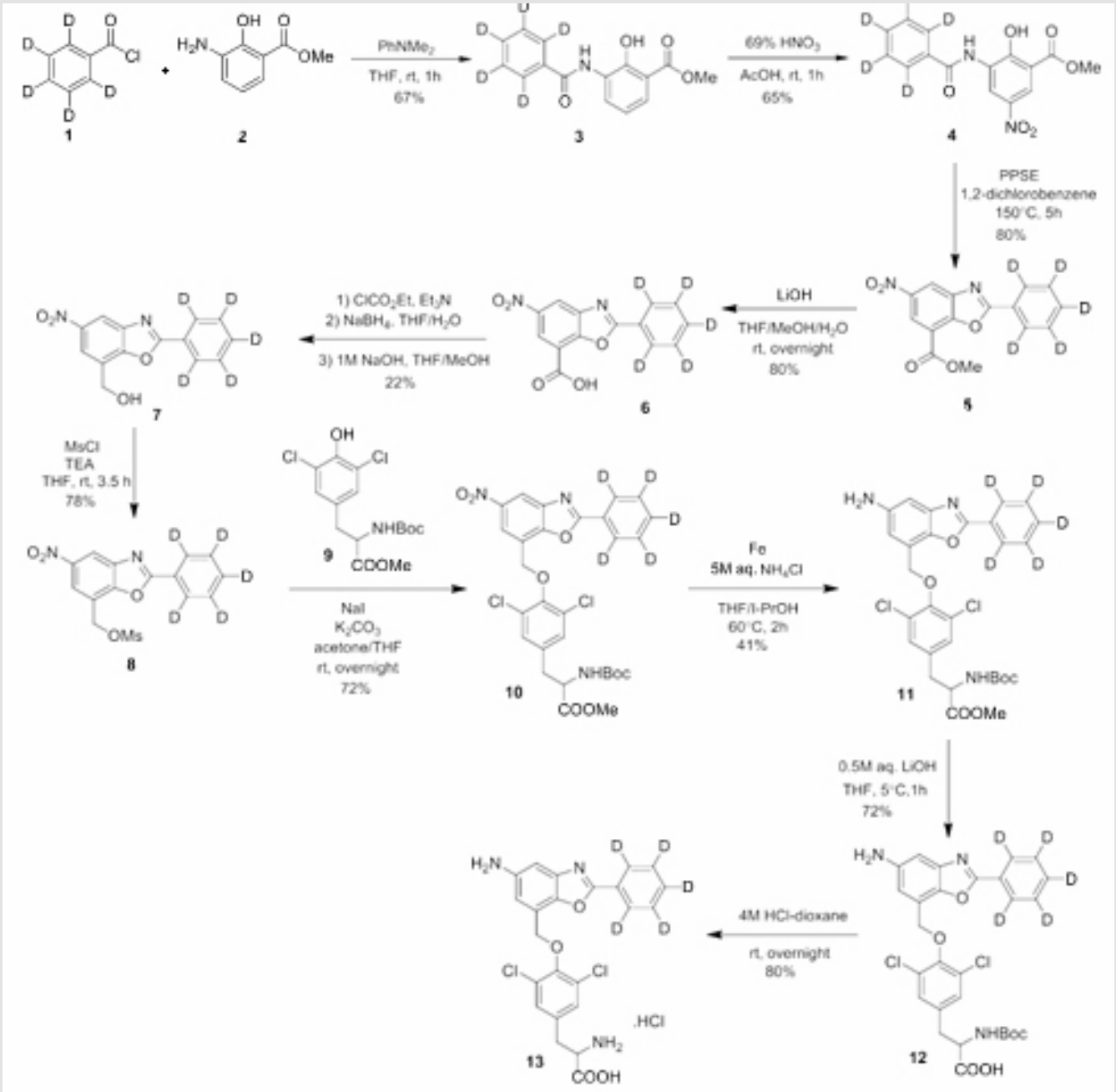
underwent a mesylation by combining the reduced acid with methane sulfonyl chloride and triethylamine in dry THF to give a 78% yield of (8). The combination of d_5-mesylate (8) with the L-tyrosine derivative (9) in a suspension of sodium iodide, potassium carbonate, and a 1:1 ratio of dry acetone and THF afforded a crude, coupled intermediate (10) in a 72% yield . The coupled product was then reduced to amine (11) by means of an iron (Fe0) catalyst in the presence of 5.0M aq. NH_4Cl to give a 41% yield. The deprotection of the methyl ester group to give acid (12) was achieved by stirring (11) with 0.5M aq. LiOH solution in THF and subsequently, the deprotection of the tert-butoxy group to give the final d_5-JPH203 (13) proceeded by adding 4.0 M HCl-1,4-dioxane to the suspended starting material in THF.



Scheme 1: Synthesis of d_5-JPH203.

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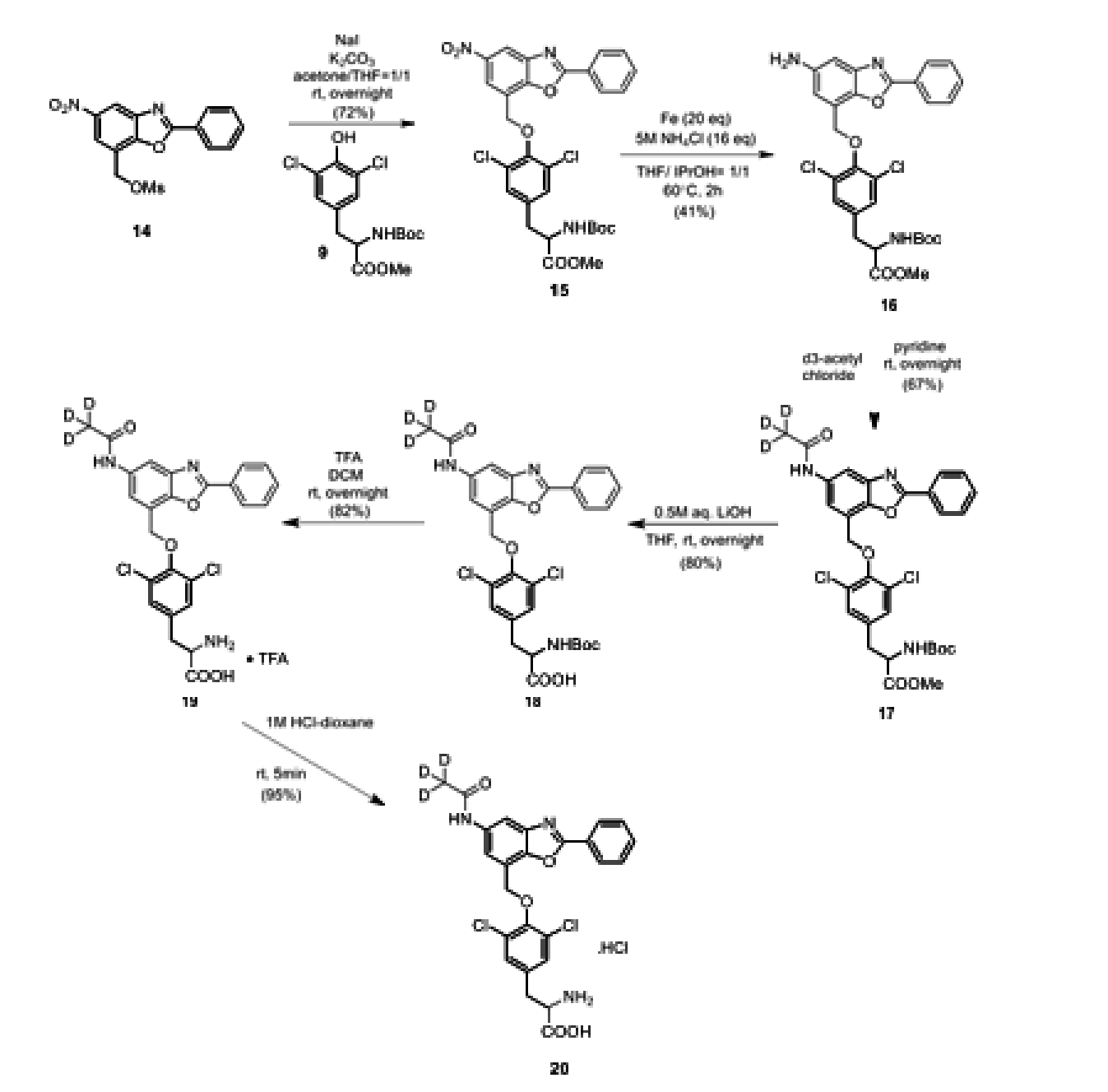

Scheme 2: Synthesis of d_3-N-acetyl JPH203.

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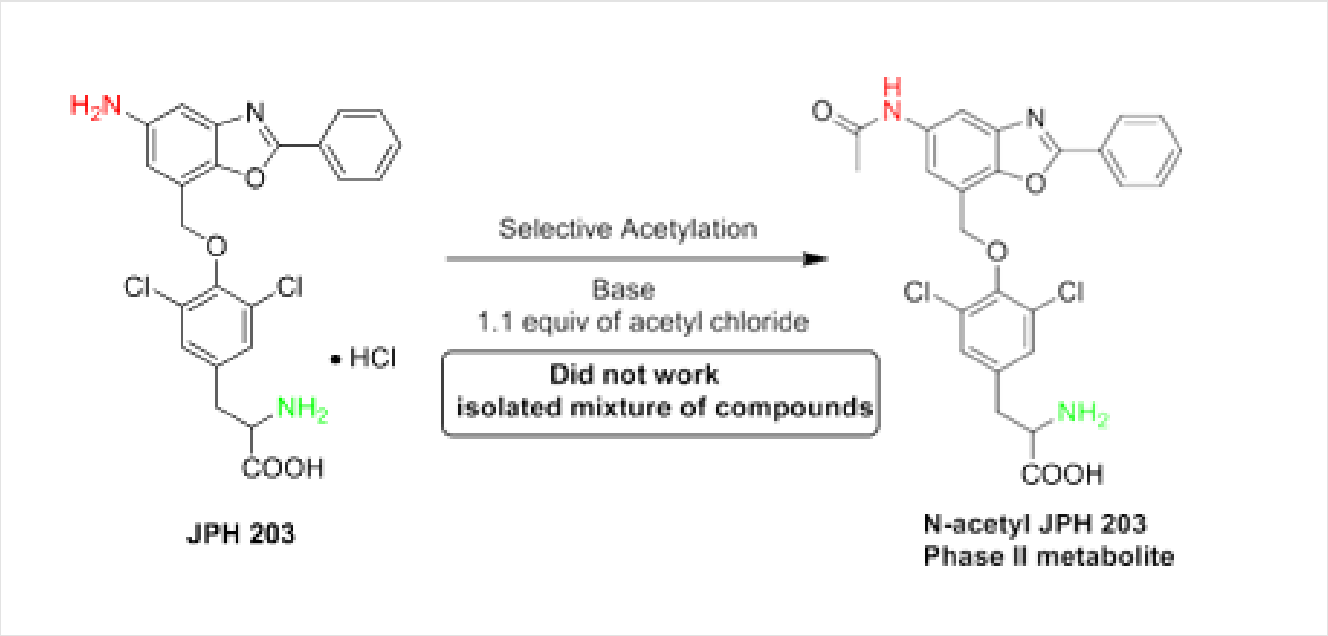

Scheme 3: Selective mono-acetylation of JPH-203.

Preparation of d_3-*N*-acetyl JPH203

The first attempt at synthesis and isolation of d_3-*N*-acetyl JPH203 combined JPH203 with equivalents of d3-acetyl chloride in the presence of a base and stirred overnight, as seen in Scheme 3. The two positions that had possible N-acetylation were at the amine on the L-tyrosine portion of the compound and on the ring of the top portion of the compound. Di-acetylation of the two positions, mono-acetylation of the amine on the amino acid, and mono-acetylation of the ring were predicted to occur during the reaction and thus required separation by purification. We tried several purifications of reaction mixture by silica gel column chromatography to afford pure mono acetylate product of the ring, but these efforts were not successful. The high polarity of the compounds prevented good separation and elution from the column. Therefore, the deuterated phase II metabolite was synthesized in similar fashion to d_5-JPH203 instead (Scheme 2). The mesylate intermediate (14) was coupled with the L-tyrosine derivative (9) to give (15) at a 72% yield. 10 The coupled compound was then reduced to an amine (16) by means of refluxing with 5.0 M aqueous NH4Cl. 10 The amine came at a 41% yield. Decreasing the reaction time from 3 hours to 2 hours gave greater yields, however 41% was the maximum yield achieved. The d_3- acetyl chloride amination of the amine group proceeded overnight at room temperature with pyridine as the base and solvent. After removal of the solvent and excess acetyl chloride, a liquid-Liquid extraction was performed to isolate the crude acetylated compound (17). This required two column chromatography purifications to get a product at a 67% yield. The following methyl ester deprotection to form (18) took longer than anticipated and thus ran overnight.

The original approach for using 4.0M HCl-dioxane in the tert-butoxy deprotection of (18) resulted in an impure form of the final product (20). The alternative approach consisted of two separate reactions deprotecting the tert-butoxy group with trifluoroacetic acid to give (19), followed by converting the compound to an HCl salt to give (20). The weaker trifluoroacetic acid results in the easy removal of the tert-butoxy group but is unlikely to remove the *N*-acetyl group. After the synthesis, LC/MS-MS methods were used to incorporate the two internal standards into the analytical method. Briefly, the method used an extend-C18 column equipped with a C18 column guard. An Applied Biosystems Sciex 4000 (Applied Biosystems; Foster City, CA) was equipped with a Shimadzu HPLC (Shimadzu Scientific Instruments, Inc.; Columbia, MD) and Nexera X2 Sil-30AC auto-sampler (Shimadzu Scientific Instruments, Inc.; Columbia, MD). The flow-rate was 0.4 ml/min and Solvent A: H2O 10 mM NH_4OAc, 0.1% Formic acid, and B: methanol:acetonitrile (1:1). The gradient conditions were as follows: 95% A for 1.0 min and then ramped to 95% B at 5.0 min and held for 3.0 min, at which point it was ramped back to 95% A at 9.0 min and held for 1.5 min (10.5 min total run time). JPH203 (472.089⟶224.126 m/z quantitative MRM; confirmatory MRM as 472.089 ⟶ 93.104 m/z) had a retention time of 5.6 min, D_5-JPH203 (479.120 ⟶ 229.212 m/z quantitative MRM; confirmatory

MRM 479.129 ⟶ 92.879 m/z) also had a retention time of 5.6 min. Whereas the Phase II metabolite *N* Acetyl-JPH203 (514.095 ⟶ 265.400 m/z major MRM; qualifier 514.095 ⟶ 222.827 m/z) had a retention time of 5.8 min, with the D_3-NAc-JPH203 (517.229 ⟶ 269.113 m/z major MRM; qualifier 517.229 ⟶ 94.161 m/z) also at 5.8 min. While the compounds contain two chlorine atoms, we did not observe cross talk in the LC/MS-MS and thus, in our hands, these are useful internal standards (IS).

Conclusion

The synthesis of d_5-JPH203 took a total of 15 steps. The linear synthesis from the salicylic acid to the final product took 12 steps, and the other three steps were involved with the synthesis of the boc-protected L-tyrosine methyl ester. The synthesis of d_3-*N*-acetyl JPH203 took a total of 18 steps, including the boc-protected L-tyrosine methyl ester synthesis. These two compounds, d_5- JPH203 and d_3-NAc-JPH203 will serve as useful internal standards for LC/MS-MS quantitative analysis of clinical samples.

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