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Facile synthesis of stable isotope-labeled antibacterial agent RWJ-416457 and its metabolite

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Synthesis of multiple stable isotope-labeled antibacterial agent RWJ-416457, (*N*-{3-[3-fluoro-4-(2-methyl-2,6-dihydro-4*H*-pyrrolo [3,4-c]pyrazol-5-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide), and its major metabolite, *N*-{3-[4-(2,6-dihydro-4*H*-pyrrolo [3,4-c]pyrazol-5-yl)-3-fluoro-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide, is described. The stable isotope-labeled [$^{13}CD_3$] RWJ-416457 was prepared readily by acetylation of the precursor amine, 5-aminomethyl-3-[3-fluoro-4-(2-methyl-2,6-dihydro-4H-pyrrolo[3,4-c]pyrazol-5-yl)-phenyl]-oxazolidin-2-one with $CD_3^{13}COCl$ in pyridine. Synthesis of the stable isotope-labeled metabolite involved a construction of multiple isotope-labeled pyrazole ring. *N,N*-dimethyl(formyl-^{13}C,D)amide dimethyl acetal was first prepared by treating *N,N*-dimethyl(formyl-^{13}C,D)amide with dimethyl sulfate, followed by sodium methoxide. Then, *N*-{3-[3-fluoro-4-(3-oxo-pyrrolidin-1-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide was condensed with *N,N*-dimethyl (formyl-^{13}C,D)amide dimethyl acetal, and the resultant β-ketoenamine intermediate underwent pyrazole ring formation with hydrazine-$^{15}N_2$, to give the [$^{13}C^{15}N_2$D]-labeled metabolite.

Keywords: isotope label; RWJ-416457; antibacterial agent; metabolite; pyrazole construction

Introduction

RWJ-416457, *N*-{3-[3-fluoro-4-(2-methyl-2,6-dihydro-4*H*-pyrrolo [3,4-c]pyrazol-5-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**1**) is an orally active antibacterial agent under investigation for the treatment of infections caused by clinically important gram-positive bacteria.[1–4] This pyrrolopyrazolyl-substituted oxazolidinone shows a high selectivity against gram-positive bacteria, as well as it demonstrates a potent in vivo efficacy. In comparison with (Food and Drug Administration) FDA-approved antibiotic Linezolid (marketed as Zyvox®), RWJ-416457 inhibited the growth of Linezolid-susceptible staphylococci, enterococci, and streptococci at concentrations of ≤4 μg/mL, generally exhibiting twofold to fourfold greater potency than that of Linezolid.[2a]

The des-methyl analog on the pyrazole ring of the parent drug, that is, *N*-{3-[4-(2,6-dihydro-4*H*-pyrrolo[3,4-c]pyrazol-5-yl)-3-fluoro-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**2**), was identified as the major metabolite. To support drug development efforts, stable isotope-labeled RWJ-416457, as well as its metabolites were required.

Stable isotope-labeled compounds are commonly used as internal standards for liquid chromatography tandem mass spectrometry (LC–MS–MS) quantitative bioanalysis of clinical and forensic samples.[5] In order to better evaluate biological behavior of drug candidates, accurate analysis is important to establish the exposure of the drug compounds in biological subjects. LC–MS has been generally used to quantify low levels of drug compounds in biological samples such as blood, plasma, serum, or urine. The nature of the internal standard is an important aspect of any quantitative analytical procedure. The optimum internal standard is a pure, stable, and isotopically labeled analog of the drug compound with a sufficiently large enough mass difference to nullify the effect of naturally abundant heavy isotopes in the drug molecule. The mass difference depends on the molecular weight and elements of the analyte. The stable isotopes should be labeled at non-exchangeable positions. The isotopically labeled compounds should be chemically pure, free or less than 0.1% of unlabeled compound, and usually possess a difference of at least 3 amu's compared with the unlabelled parent molecules.

Pyrazole is a versatile and important building block for many biologically active and pharmaceutical compounds. For example, it was built into antibacterial agents,[1–4,6,7] and other therapeutic agents such as those for treating cognitive disorder such as Alzheimer's disease.[8] There have been some literature reports for the synthesis of isotope-labeled pyrazole-containing compounds such as ^{14}C-labeled stanozolo[9] but none for the construction of multiple stable isotope-labeled pyrazole ring. Herein, we report a rapid and efficient synthesis of stable isotope-labeled antibacterial agent RWJ-416457 (**3**, Figure 1), as well as its pyrazole-containing metabolite (**4**), which involves construction of multiple stable isotope-labeled pyrazole ring.

Results and discussion

The synthesis of RWJ-416457 (**1**) and its *N*-de-methylated analog (**2**) has been reported.[3,4] The synthesis of stable isotope-labeled

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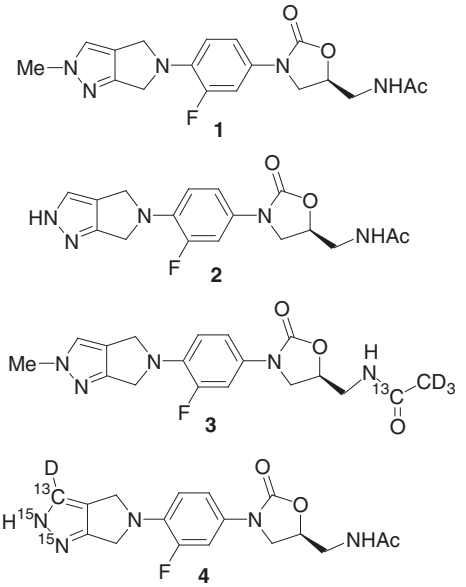


Figure 1. RWJ-416457 (**1**), its major metabolite (**2**), and their corresponding designed isotope-labeled compounds (**3** and **4**).

RWJ-416457 (**3**) is outlined in Scheme 1. Preparation of the amine precursor (**5**) was first attempted by selective hydrolysis of the acetamide group of RWJ-416457 (**1**) but failed. Instead, the amine precursor (**5**) was prepared according to general procedures described in PCT publication WO01/42242.[4] Then, [$^{13}CD_3$]RWJ-416457 was conveniently prepared by acetylation of the amine precursor, 5-aminomethyl-3-[3-fluoro-4-(2-methyl-2,6-dihydro-4H-pyrrolo[3,4-c]pyrazol-5-yl)-phenyl]-oxazolidin-2-one (**3**) with $CD_3^{13}COCl$ in pyridine in 92% isolated yield.

The synthesis of the stable isotope labeled metabolite (**4**) is shown in Scheme 2. *N*,*N*-dimethyl(formyl-^{13}C,D)amide dimethyl acetal (**8**) was first prepared in 65% yield by treating commercially available *N*,*N*-dimethyl(formyl-^{13}C,D)amide (**6**) first with dimethyl sulfate, followed by with sodium methoxide in deuterated methanol in reference to a modified literature procedure.[10] When unlabeled methanol was used as the reaction solvent in the treatment with sodium methoxide, the deuterium of the desired product (**8**, $Me_2N^{13}CD(OMe)_2$) could be substantially exchanged with proton to give $Me_2N^{13}CH(OMe)_2$ as the major product based on proton nuclear magnetic resonance (NMR) and ^{13}C-NMR analysis. Thus, deuterated methanol was employed as the solvent to avoid the Hydrogen/Deuterium (H/D) exchange.

Next, *N*-{3-[3-fluoro-4-(3-oxo-pyrrolidin-1-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**9**) was prepared according to known literature procedures.[11] Its condensation with *N*,*N*-dimethyl(formyl-^{13}C,D)amide dimethyl acetal (**8**) produced [^{13}CD]-labeled β-ketoenamine intermediate (**10**) in 54% yield. The resultant β-ketoenamine intermediate (**10**) underwent pyrazole ring formation with $^{15}N_2$-labeled hydrazine ($^{15}NH_2^{15}NH_2$) to give the [$^{13}C^{15}N_2D$]-labeled metabolite (**4**) in 55% yield. The labeled hydrazine-$^{15}N_2$ was generated in situ from commercially available hydrazine sulfate-$^{15}N_2$ ($^{15}NH_2^{15}NH_2.H_2SO_4$) and potassium t-butoxide solution in THF. Similar condensation reaction with hydrazine sulfate-$^{15}N_2$ without prior treatment with potassium t-butoxide failed to produce the desired product. Also, similar reaction with prior treatment of hydrazine sulfate-$^{15}N_2$ with two equivalents of diethyl isopropyl amine, instead of potassium t-butoxide, was unsuccessful.

An alternative synthesis for the stable isotope-labeled RWJ-416457 could be achieved by methylation of the stable isotope-labeled metabolite *N*-{3-[4-(2,6-dihydro-4*H*-pyrrolo[3,4-c]pyrazol-5-yl)-3-fluoro-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**4**) with methyl iodide following a previously reported method.[4] However, because of the known poor regioselectivity of the methylation on N1 and N2 of the pyrazole ring, as well as the difficult separation of the two methylated regioisomers this approach was not pursued.

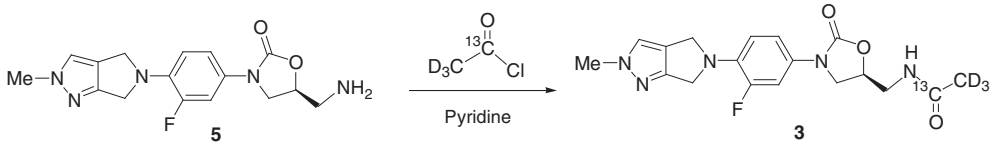
General experimental

Stable isotope-labeled reagents acetyl chloride-1-^{13}C,D_3 ($CD_3^{13}COCl$), *N*,*N*-dimethyl(formyl-^{13}C,D)amide and hydrazine sulfate-$^{15}N_2$ ($^{15}NH_2^{15}NH_2.H_2SO_4$) were purchased from Isotec (a division of Sigma-Aldrich, St. Louis, MO). Dimethyl sulfate, potassium *t*-butoxide in THF solution (1M), and deuterated methanol (CD_3OD) were purchased from Sigma-Aldrich and were used as received. Other reagents and solvents were obtained from VWR International.

Nuclear magnetic resonance spectra were acquired on a Bruker 300-Avance (300 MHz) spectrometer with Tetramethylsilane (TMS) as an internal standard. Chemical shifts are expressed in parts per million (ppm, δ scale). LC–MS analysis was performed on an Agilent 1100 series LC/MSD (Mass spectrometric detector) with an Agilent Zorbax® SB C18 column (3 μm, 2.1 × 50 mm), gradient 10–100% CH_3CN-H_2O, 0.05% TFA or 0.05% NH_4OAc over 3.5 min, hold at 100% CH_3CN for 2.5 min, flow rate at 0.5 mL/min, detection at 214 and 254 nm, and mass scan range 120–1500 amu. Flash chromatography was performed using a Teledyne Isco CombiFlash Companion system and a RediSep® silica gel (Teledyne Isco, Lincoln, Nebraska, USA) column unless otherwise specified. Reverse-phase preparative HPLC purifications were performed using a Gilson system equipped with a Phenomenex Gemini C18 column (5 μm, 21.2 × 250 mm, 110 Å) eluted at 15 mL/min with UV detection at 254 nm with a 20-min gradient from 10–90% CH_3CN in H_2O in the presence of 0.05% TFA.

5-Aminomethyl-3-[3-fluoro-4-(2-methyl-2,6-dihydro-4H-pyrrolo[3,4-c]pyrazol-5-yl)-phenyl]-oxazolidin-2-one (5)

The amine precursor (**5**) was prepared according to general procedures described in PCT publication WO01/42242.[4] MS *m/z* 332 ($M+H^+$). ^{1}H NMR ($(CD_3)_2SO$) δ 7.53 (1H, s), 7.46 (1H, dd, *J* = 15, 3 Hz), 7.17 (1H, dd, *J* = 9, 3 Hz), 6.86 (1H, t, *J* = 9 Hz), 4.57 (1H, m), 4.46 (4H, s), 4.01 (1H, t, *J* = 9 Hz), 3.84–3.79 (4H, m), 2.81 (2H, m), 1.59 (2H, s).



Scheme 1. Synthesis of stable isotope [$^{13}CD_3$]-labeled RWJ-416457, *N*-{3-[3-fluoro-4-(2-methyl-2,6-dihydro-4H-pyrrolo[3,4-c]pyrazol-5-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**3**).

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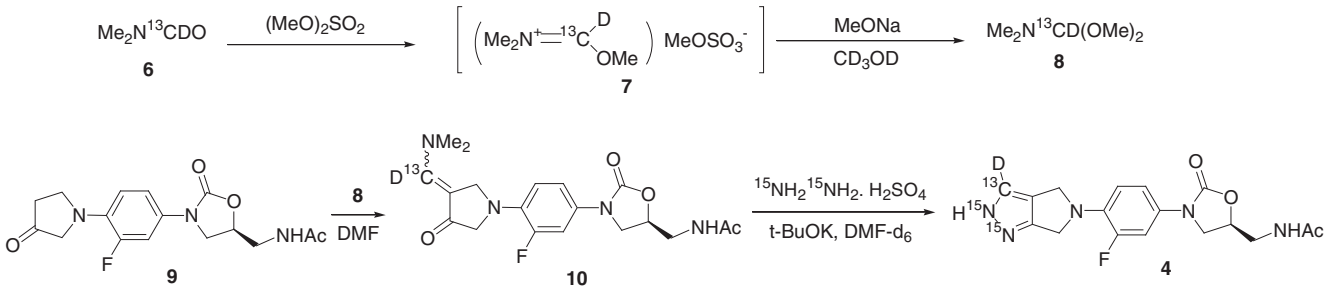

Scheme 2. Synthesis of the stable isotope [$^{13}CD^{15}N_2$]-labeled metabolite, *N*-{3-[4-(2,6-dihydro-4*H*-pyrrolo[3,4-c] pyrazol-5-yl)-3-fluoro-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**4**).

[$^{13}CD_3$]RWJ-416457 (**3**)

5-Aminomethyl-3-[3-fluoro-4-(2-methyl-2,6-dihydro-4*H*-pyrrolo [3,4-c]pyrazol-5-yl)-phenyl]-oxazolidin-2-one (**5**, 820 mg) was dissolved completely in anhydrous pyridine (15 mL) at 60°C with sonicating. The resultant solution was then cooled at room temperature and maintained as a clear solution. To this solution, a solution of $CD_3^{13}COCl$ (218 uL) in anhydrous dichloromethane (2 mL) was added. The reaction mixture was stirred at room temperature for 2 h. Crude product as a pale brown solid formed was collected by using vacuum filtration and rinsed with water to give the first batch of crude product. The filtrate was diluted with the addition of 75 mL of water to give more of crude product. The combined crude product was dissolved in a minimal amount of DMSO (~2 mL). The resultant DMSO solution was added dropwise to distilled water (15 mL) under vigorous stirring. The formed precipitate was collected by using suction filtration, rinsed with water, and dried in vacuo in the absence of light to give the desired product [$^{13}CD_3$]RWJ-416457 (**3**) as light brown solid (857 mg, 92%). MS *m/z* 378 ($M+H^+$). ^{1}H NMR ($(CD_3)_2SO$) δ 8.2 t (1H, m), 7.53 (1H, s), 7.45 (1H, dd, *J* = 15, 3 Hz), 7.15 (1H, dd, *J* = 9, 3 Hz), 6.86 (1H, t, *J* = 9 Hz), 4.70 (1H, m), 4.48 (4H, s), 4.08 (1H, t, *J* = 9 Hz), 3.85 (3H, s), 3.70 (1H, dd, *J* = 9, 6 Hz), 3.40 (2H, m). The stable isotope-labeled compound was confirmed additionally by using HPLC analysis with co-injection of unlabeled authentic RWJ-416457. Isotope analysis indicated that the labeled compound was free of unlabeled compound. The stable isotope-labeled compound was found to have a purity of 98.7 area percent by HPLC analysis using an Agilent 1200 series HPLC system with a UV detector set at 240 nm and a Waters XTerra RP18 column (Waters Corporation, MA, USA) (4.6 × 150 mm, 3.5 μM) and a gradient of acetonitrile in water in the presence of 0.05% TFA by volume at flow rate 1 mL/min and column temperature at 25°C.

N,N-Dimethyl(formyl-^{13}C,D)amide dimethyl acetal (**8**)

Synthesis of the labeled DMF dimethyl acetal was referred to a modified literature procedure.[10]

(1) Dimethyl *N,N*-dimethyl(formyl-^{13}C,D)amide-sulfate (**7**)

In a round-bottom flask (500 mL) under nitrogen, anhydrous *N,N*-dimethyl(formyl-^{13}C,D)amide (**6**, 5.0 g, 66.67 mmol) and dimethyl sulfate (8.41 g, 6.31 ml, 1 eq) were added. The mixture was then heated at 80°C for 3 h. Then, after cooling at room temperature, it was added to the reaction mixture of an equal volume of anhydrous diethyl ether (~12 mL). The solvent was evaporated and the oily residue (**7**, quantitative yield) was used as it was in the next step.

(2) *N,N*-Dimethyl(formyl-^{13}C,D)amide dimethyl acetal (8)

To a dried round-bottom flask under nitrogen, sodium methoxide (3.6 g, 66.67 mmol, 1 eq) and 21 mL of anhydrous deuterated methanol (CD_3OD) was added. This solution was cooled down to −10°C and at −10°C under nitrogen, was added dropwise the aforementioned complex (**7**) in a period of 1.5 h. When the addition was complete, stirring was continued for another 2 h. The mixture was then distilled slowly at atmospheric pressure in an oil bath gradually heated from 70 to 90°C and various fractions were collected into a round-bottom flask containing magnesium sulfate. Once the residue solid was dry, the liquid-containing product was then slowly distilled using a vigreux long distillation column in order to eliminate methanol solvent. Fractions containing the products (b.p. 105–106°C/760 mmHg) were collected (5.62 g, 65%). ^{1}H NMR ($CDCl_3$) δ 3.34 and 3.32 (each 3H, s), 2.29 and 2.28 (each 3H, s).

N-{3-[3-Fluoro-4-(3-oxo-pyrrolidin-1-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**9**)

This intermediate was prepared according to the procedures described on the literature.[11] MS *m/z* 336 ($M+H^+$).

[^{13}CD]*N*-{3-[4-(3-Dimethylaminomethylene-4-oxo-pyrrolidin-1-yl)-3-fluoro-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**10**)

To a round-bottom flask was charged with the aforementioned intermediate ketone[11] (**9**, 761 mg, 2.28 mmol) and anhydrous DMF (2.3 mL). The mixture was heated in an oil bath at 60°C with agitation to achieve a clear dissolution. To this solution, the aforementioned prepared *N,N*-dimethyl(formyl-^{13}C,D)amide dimethyl acetal (**8**, 1.9 mL, 6.4 eq) under nitrogen atmosphere was added. The mixture was heated in an oil bath at 60°C and agitated for 1.5 h. The reaction was cooled at room temperature, and then heptane (5 mL) was added with fast agitation for 5 min. The brown solid was filtered, washed with heptane (5 mL × 2), and dried by air suction. The resulting solid was dried at 40°C under vacuum overnight. The intermediate was obtained as a brown powder (482 mg, ~54%), which was used in the next reaction without further purification. MS analysis confirmed the identity of the compound (MS *m/z* 393 ($M+H^+$)).

[$^{13}C^{15}N_2D$]*N*-{3-[4-(2,6-Dihydro-4*H*-pyrrolo[3,4-c]pyrazol-5-yl)-3-fluoro-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (4)

The aforementioned labeled enamine intermediate **(10)** (435 mg, 1.11 mmol) was dissolved in DMF-d_6 (2.2 mL) in a round-bottom flask heated in an oil bath at 90°C and with agitation. Hydrazine sulfate-$^{15}N_2$ ($^{15}NH_2^{15}NH_2.H_2SO_4$) (179.2 mg, 1.35 mmol) in another round-bottom flask was dissolved in 1.1 mL of D_2O in a warm bath with sonication. To this solution, 1.34 mL of 1M potassium t-butoxide in THF solution was added. The resultant solution was then added to the aforementioned enamine solution. This reaction mixture was then heated in an oil bath at 90°C and stirred under nitrogen for 6 h until TLC analysis indicated the completion of the reaction. The reaction mixture was cooled at room temperature and added dropwise into distilled water (20 mL) with vigorous stirring. The suspension mixture was stirred at room temperature for 20 min and was allowed to stand for 20 min and the solid was isolated by using filtration. This solid was dried by using suction filtration and then placed in a vacuum oven at room temperature overnight to give the crude product as a light brown solid.

The crude product was dissolved in a minimal amount of DMSO (~1.5 mL), and filtrated to give a clear solution. The resultant DMSO solution was added dropwise to distilled water (15 mL) under vigorous stirring. The formed precipitate was collected by using suction filtration, rinsed with water, and dried in vacuo in the absence of light. The precipitation procedure was repeated second time to give the desired product as a light brown solid (222 mg, 55%). MS *m/z* 364 ($M+H^+$). ^1H NMR (($CD_3)_2SO$) \ 12.66 (1H, d, br, $J=108$ Hz, $H^{15}N$), 8.25 (1H, t), 7.48 (1H, dd, $J=15$, 3 Hz), 7.15 (1H, dd, $J=9$, 3 Hz), 6.88 (1H, t, $J=9$ Hz), 4.70 (1H, m), 4.48 (4H, s), 4.10 (1H, t), 3.70 (1H, t), 3.40 (2H, t), 1.85 (3H, s). The stable isotope-labeled compound was confirmed additionally by HPLC analysis with co-injection of unlabeled authentic compound. The isotope analysis indicated that the labeled compound was free of unlabeled compound. HPLC analysis showed the prepared stable isotope-labeled compound to have a chromatographic purity of 97.4 area percent using a UV detector set at 240 nm, a Waters Xterra RP18 C18 column (4.6 × 150 mm, 3.5 μM), with column temperature at 25°C and a gradient of acetonitrile in water in the presence of 0.05% TFA by volume, at flow rate 1 mL/mL.

Conclusion

A facile and efficient synthesis of stable isotope-labeled antibacterial agent RWJ-416457, (**3**, *N*-{3-[3-fluoro-4-(2-methyl-2,6-dihydro-4*H*-pyrrolo[3,4-c]pyrazol-5-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide), and its major metabolite, *N*-{3-[4-(2,6-dihydro-4*H*-pyrrolo[3,4-c]pyrazol-5-yl)-3-fluoro-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**4**), is described. [$^{13}CD_3$]-labeled RWJ-416457 was conveniently prepared by acetylation of the precursor amine, 5-aminomethyl-3-[3-fluoro-4-(2-methyl-2,6-dihydro-4*H*-pyrrolo[3,4-c]pyrazol-5-yl)-phenyl]-oxazolidin-2-one (**5**) with $CD_3^{13}COCl$ in pyridine. Key steps for the synthesis of the stable isotope-labeled metabolite (**4**) involved the condensation of *N*-{3-[3-fluoro-4-(3-oxo-pyrrolidin-1-yl)-phenyl]-2-oxo-oxazolidin-5-ylmethyl}-acetamide (**9**) with *N,N*-dimethyl (formyl-^{13}C,D)amide dimethyl acetal (**8**), which was prepared by treating *N,N*-dimethyl(formyl-^{13}C,D)amide (**6**) with dimethyl sulfate, followed by sodium methoxide to give *β*-ketoenamine intermediate (**10**) and subsequent pyrazole ring formation with ^{15}N-labeled hydrazine $^{15}NH_2^{15}NH_2$. The synthesis of the pyrazole-containing metabolite, involved the construction of multiple stable isotope-labeled pyrazole ring from ketone compound, provides a general and convenient synthetic method for multiple stable isotope labeling synthesis of biologically and pharmaceutically important pyrazole compounds.

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Conflict of Interest

The authors did not report any conflict of interest.

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