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Synthesis of [1-^{13}C-5-^{12}C]-alpha-ketoglutarate enables non-invasive detection of 2-hydroxyglutarate

Natsuko Miura[#1], **Chandrasekhar Mushti**[#2], **Deepak Sail**[2], **Jenna E. AbuSalim**[1,3], **Kazutoshi Yamamoto**[4], **Jeffrey R. Brender**[4], **Tomohiro Seki**[4], **Deyaa I. AbuSalim**[5], **Shingo Matsumoto**[4], **Kevin A. Camphausen**[1], **Murali C. Krishna**[4], **Rolf E. Swenson**[2], **Aparna H. Kesarwala**[1,3]

[1]Radiation Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[2]Chemistry and Synthesis Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

[3]Department of Radiation Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA, USA

[4]Radiation Biology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[5]Department of Chemistry, Indiana University, Bloomington, IN, USA

[#] These authors contributed equally to this work.

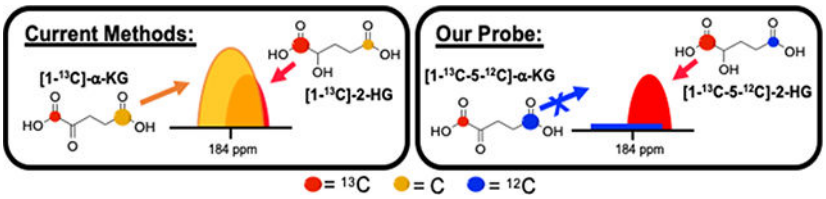
Abstract Summary:

Isocitrate dehydrogenase 1 (IDH1) mutations that generate the oncometabolite 2-hydroxyglutarate (2-HG) from α-ketoglutarate (α-KG) have been identified in many types of tumors and are an important prognostic factor in gliomas. 2-hydroxyglutarate production can be determined by hyperpolarized carbon-13 magnetic resonance spectroscopy (HP-^{13}C-MRS) using [1-^{13}C]-α- KG as a probe, but peak contamination from naturally-occurring [5-^{13}C]-α-KG overlaps with the [1-^{13}C]-2-HG peak. Via a newly developed oxidative-Stetter reaction, [1-^{13}C-5-^{12}C]-α-KG was synthesized. α-KG metabolism was measured via HP-^{13}C-MRS using [1-^{13}C-5-^{12}C]-α-KG as a probe. [1-^{13}C-5-^{12}C]-α-KG was synthesized in high yields, and successfully eliminated the signal from C5 of α-KG in the HP-^{13}C-MRS spectra. In HCT116 IDH1 R132H cells, [1-^{13}C-5-^{12}C]-α-KG allowed for unimpeded detection of [1-^{13}C]-2-HG. ^{12}C-enrichment represents a novel method to circumvent spectral overlap, and [1-^{13}C-5-^{12}C]-α-KG shows promise as a probe to study IDH1 mutant tumors and α-KG metabolism.

Graphical Abstract

Corresponding author: Aparna H. Kesarwala, MD, PhD, Department of Radiation Oncology, Winship Cancer Institute, Emory University School of Medicine, 1365 Clifton Road NE, Atlanta, Georgia 30322, aparna.kesarwala@emory.edu.

Conflicts of interest:
Work as presented in this manuscript has been filed under a pending patent application.



2-hydroxyglutarate production can be determined by HP-^{13}C-MRS using [1-^{13}C]-α- KG, but contamination from naturally-occurring [5-^{13}C]-α-KG overlaps with the [1-^{13}C]-2-HG peak. We synthesized [1-^{13}C-5-^{12}C]-α-KG via a newly developed oxidative-Stetter reaction and eliminated the signal from C5 of α-KG in the HP-^{13}C-MRS spectra, thereby representing a novel method to circumvent spectral overlap.

Keywords

Hyperpolarized carbon-13 nuclear magnetic resonance spectroscopy; alpha-ketoglutarate; Isocitrate dehydrogenase 1; 2-hydroxyglutarate; Carbon-13 labeled metabolites

Introduction:

Atypical metabolism of alpha-ketoglutarate (α-KG) has been linked to increased tumor cell differentiation,[1] amplified malignant progression,[1] as well as altered protein synthesis and catabolism.[2] α-KG is also the substrate for the mutant isocitrate dehydrogenase I (IDH1) enzyme. IDH1 is a cytosolic enzyme that catalyzes the oxidation of isocitrate to α-KG. Mutations in this enzyme, most commonly a heterozygous point mutation of arginine 132 to histidine (R132H), instead allow for the reduction of α-KG to 2-hydroxyglutarate (2-HG).[3] This gain-of-function mutation leads to a buildup of 2-HG in IDH1 mutant cells.[3] As pyruvate and α-KG are structurally similar, 2-HG can also be produced through non-canonical activity of lactate dehydrogenase (LDH), but these promiscuous reactions by LDH do not typically lead to high concentrations of 2-HG in human tissues.[4,5] As 2-HG acts as an inhibitor of α-KG-dependent dioxygenases,[6] the high concentrations of 2-HG in IDH1 mutant cells can have multiple downstream effects.[6]

Roughly 80% of grade II and III gliomas and secondary glioblastomas contain mutations in IDH1.[7] Similar mutations have been found in other tumor types, including acute myeloid leukemia,[8,9] chondrosarcoma,[10] and intrahepatic cholangiocarcinoma[11] as well as in colorectal and pancreatic adenocarcinomas.[12,13] As IDH1 R132H mutations correlate with hypermethylation,[14] increased radiosensitivity,[15,16] and a less aggressive phenotype,[15,17] IDH1 status has become important for patient stratification and tumor classification.[18,19]

In the absence of a gain of function mutation in IDH1, concentrations of 2-HG are typically below the levels detectable by magnetic resonance spectroscopy (MRS) methods, which allows for the high concentrations of 2-HG in IDH1 mutant cells to be a unique indicator of IDH1 status *in vivo*.[20] MRS is a technique that can be used to measure 2-HG production in both biopsy samples[21] and in patients.[21,22] While *in vivo* ^{1}H MRS in principle offers a method for measuring 2-HG levels, overlapping resonances[23] and the general insensitivity of ^{1}H MRS has made accurate quantitation difficult.[24]

In 2013, Chaumeil and colleagues developed a complementary approach, measuring the real-time metabolism of α-KG to 2-HG and glutamate by injecting [1-^{13}C]-α-KG into rats bearing glioblastoma xenografts.[25] When mutant IDH1 is present, [1-^{13}C]-2-HG is produced and can be measured via hyperpolarized carbon-13 MRS (HP-^{13}C-MRS). HP-^{13}C-MRS has become an important tool in the study of real-time metabolism *in vivo*, as hyperpolarization allows for an over 10,000-fold enhancement of MRI signal of ^{13}C labeled molecules.[26] To perform HP-^{13}C-MRS studies, a highly-concentrated solution of the desired metabolite is cooled by liquid helium, polarized using a super-conducting magnet, and subsequently rapidly dissolved in a pH neutralizing solution.[27] The hyperpolarized sample can then be injected either *in vitro* or *in vivo*, and metabolism of the sample can be followed by MRI.[27] Currently, HP-^{13}C-MRS is being used to track lactate production from injected hyperpolarized [1-^{13}C]-pyruvate to non-invasively diagnose cancer, image tumor location, and monitor response to therapy in patients with prostate cancer.[28,29] Additionally, HP-^{13}C-MRS is being developed for use in various other tumor types.[29]

Unfortunately, when using [1-^{13}C]-α-KG, the HP-^{13}C-MRS signal from the naturally-present ^{13}C at the C5 position of [1-^{13}C]-α-KG (184.0 ppm) overlaps with C1 of [1-^{13}C]-2-HG (183.9 ppm) produced by IDH1 mutant enzyme (Figure 1A).[25] This makes the detection of [1-^{13}C]-2-HG and therefore characterization of IDH1 status difficult. We hypothesized that ^{12}C enrichment of C5 of α-KG would eliminate the peak contamination from the naturally-occurring [5-^{13}C]-α-KG and allow for unimpeded detection of [1-^{13}C]-2-HG via HP-^{13}C-MRS (Figure 1B). Consequently, we synthesized [1-^{13}C-5-^{12}C]-α-KG to test its utility as a probe for non-invasive *in vivo* characterization of IDH1 status and overall α-KG metabolism.

Experimental:

Chemicals and reagents

2-HG, α-KG, L-glutamate (Glu), [1-^{13}C]-α-KG, *N*-acetyl-glutamine (NAG), ammonium acetate, ammonium hydroxide and UPLC/MS grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified through a Milli-Q Integral 5 system supplied by EMD Millipore (Billerica, MA). See SI for further details on the synthesis of all substrates and ^{13}C,^{12}C-bis-labeled compounds.

Synthesis of [1-^{13}C-5-^{12}C]-alpha-ketoglutarate

Our synthesis of [1-^{13}C-5-^{12}C]-α-KG is outlined in Figure 2 and detailed in the SI. Briefly, we synthesized substrates 2-Bromo-1-morpholinoethan-1-one-1-^{13}C **(6)** and Benzyl acrylate-1-^{12}C **(7)** using standard methods. We then optimized a one-pot oxidation-Stetter reaction to form Benzyl 5-morpholino-4,5-dioxopentanoate-1-^{12}C-5-^{13}C **(8)** without detection of benzoin product **(9)**. We converted **(8)** into the bis-isopropyl ester Diisopropyl 2-oxopentanedioate-5-^{12}C-1-^{13}C **(10)** and then de-esterified to form [1-^{13}C-5-^{12}C]-α-KG (**11**).

Cell culture

HCT116 IDH1 R132H cells were purchased from Horizon Discovery (Cambridge, United Kingdom). Cells were cultured at 37 °C under 5% CO_2 in RPMI medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Reagent preparation

All α-KG derivatives were dissolved in D_2O: D-glycerol = 1:1 containing 17.3 mM of Ox063 at a concentration of 5.9 M. After three freeze and thaw cycles using liquid nitrogen followed by vortexing for 1 min, samples were stored at 4°C until use.

^{13}C MRI of hyperpolarized ^{13}C-labeled α-KG

For all hyperpolarization experiments, 35 μL of Sigma [1-^{13}C]-α-KG or [1-^{13}C-5-^{12}C]-α-KG solution containing 2.5 mM of gadolinium chelate (ProHance, Bracco Diagnostics, Milano, Italy) was polarized at 3.35 T and 1.45–1.5 K in a Hypersense DNP polarizer (Oxford Instruments, Abingdon, UK) for 3–5 h according to the manufacturer's instructions. The polarized samples were rapidly dissolved in 4.0 mL of alkaline buffer containing 25 mM Tris(hydroxymethyl)aminomethane, 50 mg/L ethylendiaminetetraacetic acid, and 37.5 mM NaOH, for the final dissolution buffer to be pH 7.4 after mixture with α-KG. For *in vitro* experiments, the hyperpolarized ^{13}C-α-KG solution (1 mL) was injected into a glass vial (Wheaton Science Products, Millville, NJ) placed in a 3T scanner (MR Solutions, Guildford, UK) via a plastic tube using a 17-mm custom-build ^{13}C solenoid leg coil placed inside of a saddle coil for ^{1}H.

Preparation for HP-^{13}C-MRS with enzymes

1.5 mL of a solution of 300 mM NaCl, 40 mM Tris–HCl pH 7.5, 20 mM MgCl2, and 0.06% bovine serum albumin were prepared in a glass vial. After shimming, 75 μL of 10 mM NADH and 500 μL of 5 kU/mL of LDH (porcine heart LDH, Sigma) in PBS were added, mixed and shimmed again immediately before measurement.

Preparation of cells for in vitro HP-^{13}C-MRS

Cells harvested by trypsinization 24 hr after plating, washed with serum-free DMEM without pyruvate and resuspended in serum-free DMEM without pyruvate with the concentration of 5.0×10^7 cells/mL. After pre-warmed to 37 °C, 2 mL of cell suspension (1.0×10^8 cells total) were transferred to a glass vial immediately before measurement.

Results:

Initially, we synthesized [1-^{13}C-5-^{12}C]-α-KG following a procedure reported by Baldwin using ^{12}C-benzylacrylate (**6**) as well as the ^{13}C-labeled nitromethane.[30] We noted that several steps were low yielding and not scalable, so we sought a more robust synthetic route. Our final complete synthesis of [1-^{13}C-5-^{12}C]-α-KG, as outlined in Figure 2, was run on gram scale with an overall yield of 88% over 3 steps. The ^{13}C spectrum of [1-^{13}C-5-^{12}C]-α-KG can be seen in Figure 3.

Building on previous work by Stetter,[31] Rovis,[32] and Yamaguchi,[33] we devised a synthetic approach that is both high-yielding and scalable. In the Stetter reaction, thiazolium salt (**2**) catalyzes the addition of a glyoxylpyrrolidide (**1**) to α,β-unsaturated carbonyl (**3**) to form α-ketoglutarate derivative (**4**) (Figure 2A). Rovis and colleagues further refined this reaction, demonstrating that glyoxylmorpholides (**5**) proceed with better yields than glyoxylpyrrolidides (**1**).[32] Glyoxylamides (e.g. **1** and **5**) are air and water sensitive, and present challenges in synthesis and isolation.[32]

To circumvent these problems, we chose to incorporate the mild N-hydroxy-4-N,N-dimethylaminopyridine, *in-situ* oxidation of 1-^{13}C-2-bromo-morpholino-acetamide (**6**) to the desired glyoxylamide (**5**) developed by Yamaguchi[33] that was used for the subsequent Stetter reaction (Figure 2B). This one-pot oxidation-Stetter reaction was found to efficiently prepare the ^{13}C-^{12}C-bislabeled products, forming **8** in 90% yield (Figure 2B). The lack of benzoin product **(9)** resulting from the self-condensation of glyoxyamides implies that the rate of oxidation of the bromo-acetamide (**6**) to the intermediate glyoxylamide (**5**) was slow, and when coupled to an efficient Michael addition, the resulting low concentrations of the glyoxylamide made self-condensation unfeasible. The coupling of a slow oxidation to an efficient Michael addition should be useful in other challenging Stetter reactions.

To determine whether [1-^{13}C-5-^{12}C]-α-KG would successfully eliminate the C5 peak in the HP-^{13}C-MRS, signals from hyperpolarized [1-^{13}C-]-α-KG and [1-^{13}C-5-^{12}C]-α-KG were compared. The hyperpolarized [1-^{13}C-5-^{12}C]-α-KG had a sufficient lifetime with a calculated T1 of 43.4 ± 0.3 seconds at 3 Tesla. When [1-^{13}C-]-α-KG was hyperpolarized, a peak corresponding to C5 of α-KG ([5-^{13}C]-α-KG) was clearly monitored at 184 ppm (Figure 4A), but there was no comparable peak at 184 ppm for hyperpolarized [1-^{13}C-5-^{12}C]-α-KG (Figure 4B). The disappearance of this signal suggests that ^{12}C enrichment of the C5 position of α-KG successfully eliminated the signal from the naturally abundant ^{13}C at this position, which is more clearly visible in Figure 4C. In both [1-^{13}C-]-α-KG and [1-^{13}C-5-^{12}C]-α-KG spectra, C1-hydrate, and C2 peaks were identified at 182 and 208 ppm, respectively. Under hyperpolarization conditions, cyclization of both [1-^{13}C]-α-KG and [1-^{13}C-5-^{12}C]-α-KG occurred, as seen by the peaks at 163 and 209 ppm in both phantom spectra, corresponding to C1 and C2 of the cyclized product respectively (Figures 4A–B). The [1-^{13}C]-α-KG phantom spectra also included a peak at 175 ppm which corresponds to C5 of the cyclized product.

With the spectral signal corresponding to C5 of α-KG eliminated, we tested the utility of [1-^{13}C-5-^{12}C]-α-KG as a probe to measure α-KG metabolism. As lactate dehydrogenase can catalyze the conversion of α-KG to 2-HG via a non-canonical reaction,[4] we utilized purified lactate dehydrogenase as proof of principle for the conversion of [1-^{13}C-5-^{12}C]-α-KG. Using LDH enzymes supplemented with NADH, [1-^{13}C-5-^{12}C]-α-KG was rapidly metabolized to [1-^{13}C]-2-HG, and a peak at 184 ppm could be clearly detected with HP-^{13}C-MRS (Figure 4D).

After confirming that [1-^{13}C-5-^{12}C]-α-KG allows for detection of [1-^{13}C]-2-HG using LDH enzymes, we tested this probe *in cellulo*. The metabolism of α-KG in HCT116 IDH1 R132H cells was monitored by adding hyperpolarized [1-^{13}C-5-^{12}C]-α-KG directly

before the HP-^{13}C-MRS measurements. In IDH1 R132H cells, [1-^{13}C]-2-HG was detected at 184 ppm without interference from [5-^{13}C]-α-KG (Figure 4E). A peak at 163 ppm was also seen, which is likely due to overlapping signals from C1 of the cyclic lactone form of [1-^{13}C-5-^{12}C]-α-KG and [1-^{13}C]-bicarbonate formed during the decarboxylation of [1-^{13}C-5-^{12}C]-α-KG by α-ketoglutarate dehydrogenase. The unidentified metabolite at 175 ppm is consistent with previously published literature.[25,34]

Discussion:

These results demonstrate [1-^{13}C-5-^{12}C]-α-KG as a probe for non-invasive imaging of IDH1 status. We developed an oxidation-Stetter reaction to streamline the synthesis of labeled α-KG. This reaction was high yielding, and benzoin side-products were not formed. Our total synthetic route was easily scalable and allowed for production of [1-^{13}C-5-^{12}C]-α-KG on gram scale and in high yields.

By removing peak-contamination from the naturally-occurring [5-^{13}C]-α-KG, the [1-^{13}C]-2-HG produced in IDH1 mutant cells could be detected cleanly *in cellulo* using [1-^{13}C-5-^{12}C]-α- KG. Though [1-^{13}C-5-^{12}C]-α-KG allowed for the classification of IDH1 status via detection of [1-^{13}C]-2-HG *in cellulo*, the low cell permeability of α-KG led to low concentrations of labeled [1-^{13}C]-2-HG. In 2007, MacKenzie *et al.* demonstrated that esters of α-KG had more favorable permeability than α-KG, where introduction of α-ketoglutarate derivatives increased prolyl hydroxylase (PHD) activity and HIF1α levels *in vitro*.[35] Esterification of our probe might therefore increase intracellular concentrations of [1-^{13}C]-2-HG and improve detection of the metabolite, as well as allow for tracking of overall α-KG metabolism *in vivo*.

With the successful use of hyperpolarized [1-^{13}C-5-^{12}C]-α-KG in tracking 2-HG production, we hypothesize that this probe can be a tool to study α-KG metabolism in a broad range of applications. Further study of these probes is warranted for transition into clinical use, but [1-^{13}C-5-^{12}C]-α-KG shows promise as a probe to study α-KG metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations:

α-KG	α-ketoglutarate
2-HG	2-hydroxyglutarate
HP	Hyperpolarized
IDH1	Isocitrate dehydrogenase
LDH	Lactate dehydrogenase
PHD	Prolyl hydroxylase

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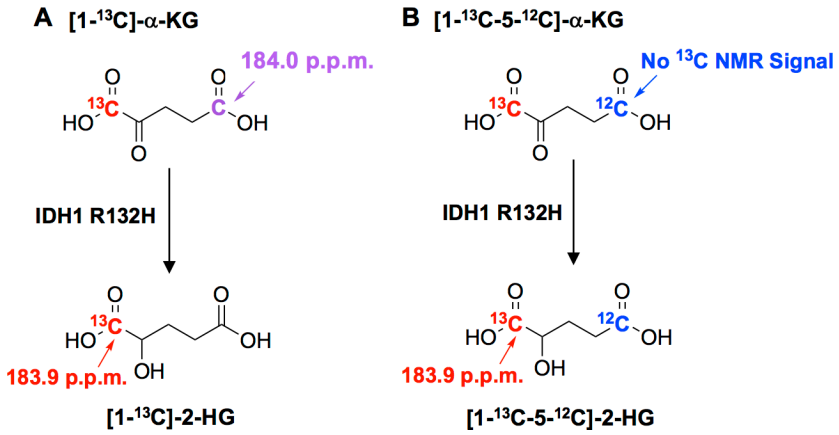

Figure 1: Peak contamination from the naturally abundant ^{13}C of C5 of α-KG and synthesis of [1-^{13}C-5-^{12}C]-α-KG

IDH1 R132H enzymes can catalyze the conversion of [1-^{13}C]-α-KG (**A**) or [1-^{13}C-5-^{12}C]-α -KG (**B**) to [1-^{13}C]-2-HG or [1-^{13}C-5-^{12}C]-2-HG respectively.

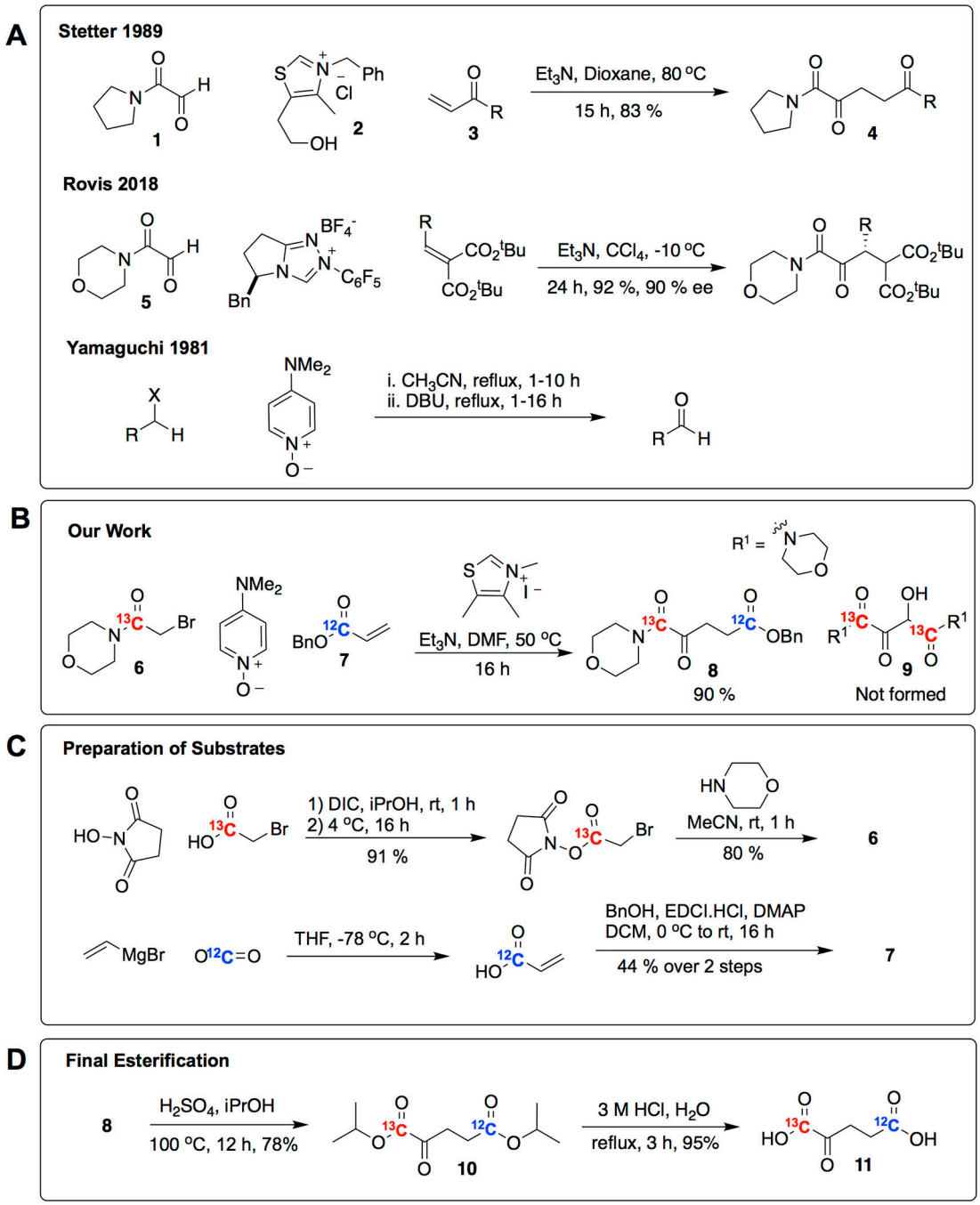

Figure 2: Background and synthesis of [1-^{13}C-5-^{12}C]-α-KG

(**A**) Key aspects of the Stetter, Rovis and Yamaguchi reactions were used to create the one-pot oxidation-Stetter reaction (**B**) we developed to streamline the synthesis of [1-^{13}C-5-^{12}C]-α KG. (**C**) Standard synthetic methods were used to reach the precursors of our oxidation-Stetter reaction. (**D**) The final steps of the synthesis of [1-^{13}C-5-^{12}C]-α-KG.

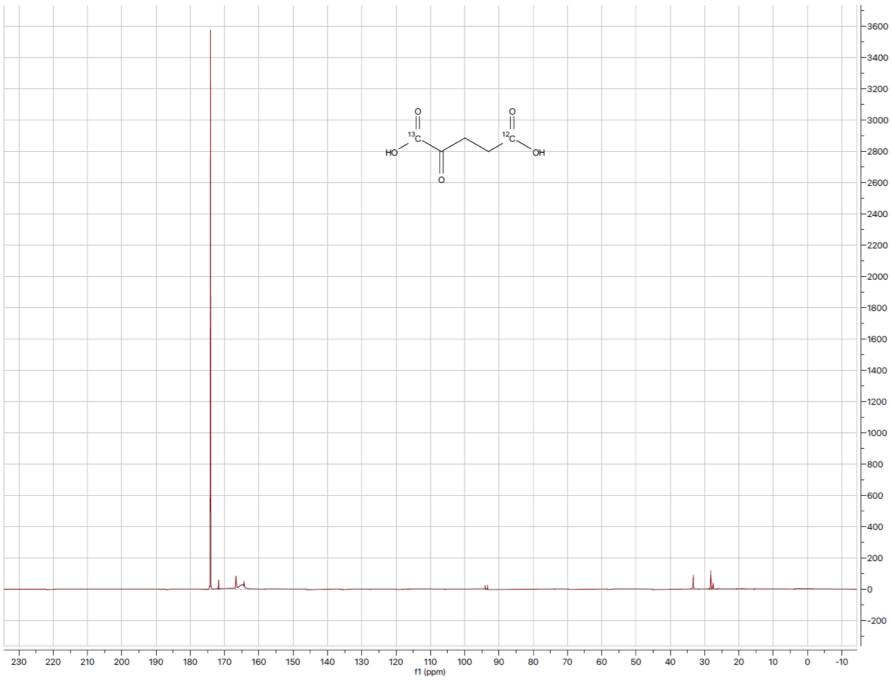

Figure 3:
^{13}C spectrum of [1-^{13}C-5-^{12}C]-α-KG

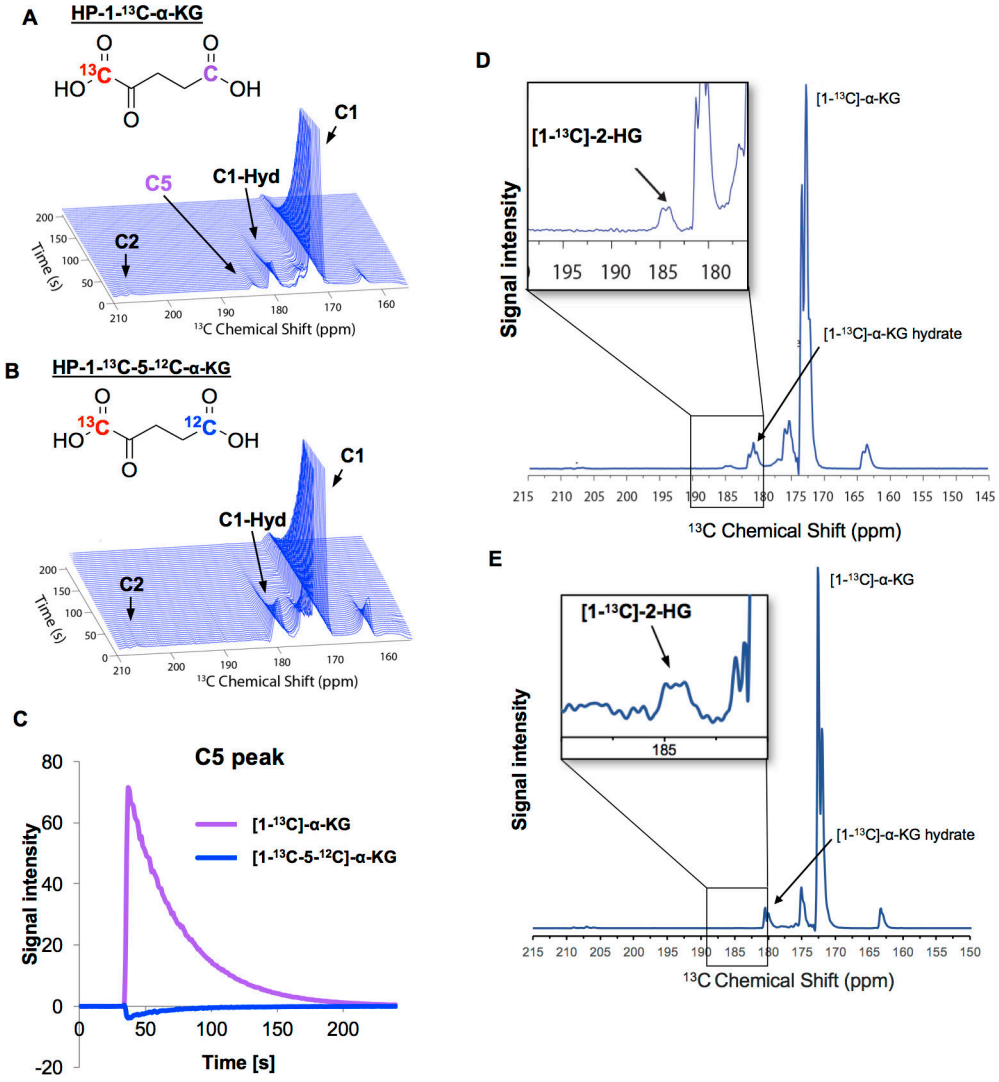

Figure 4: Elimination of C5 peak and detection of [1-^{13}C]-2-HG by HP-^{13}C-MRS

(A) Hyperpolarization of commercial [1-^{13}C]-α-KG. **(B)** Hyperpolarization of the newly synthesized [1-^{13}C-5-^{12}C]-α-KG. (**C)** Time-dependent changes in peaks detected in C5 position of α-KG. Purple and Blue lines: changes in peak heights at the position of 184 ppm in after injection of [1-^{13}C]-α-KG and [1-^{13}C-5-^{12}C]-α-KG, respectively. (**D)** Representative spectrum from purified LDH enzymes after injecting with [1-^{13}C-5-^{12}C]-α-KG. HP-^{13}C-MRS was magnified at 185 ppm for better visualization of [1-^{13}C]-2-HG. **(E)** Representative spectra from HCT116 IDH1 R132H cells after injecting with [1-^{13}C-5-^{12}C]-α-KG. HP-^{13}C-MRS was magnified at 185 ppm for better visualization of [1-^{13}C]-2-HG. N=2 biological replicates.