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Hyperpolarized Sodium [1-¹³C]-Glycerate as a Probe for Assessing Glycolysis In Vivo

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Abstract

Hyperpolarized ¹³C magnetic resonance spectroscopy (MRS) provides unprecedented opportunities to obtain clinical diagnostic information through in vivo monitoring of metabolic pathways. The continuing advancement of this field relies on the identification of molecular probes that can effectively interrogate pathways critical to disease. In this report, we describe the synthesis, development, and in vivo application of sodium [1-¹³C]-glycerate ([¹³C]-Glyc) as a novel probe for evaluating glycolysis using hyperpolarized ¹³C MRS. This agent was prepared by a concise synthetic route and formulated for dynamic nuclear polarization. [¹³C]-Glyc displayed a high level of polarization and long spin–lattice relaxation time—both of which are necessary for future clinical investigations. In vivo spectroscopic studies with hyperpolarized [¹³C]-Glyc in rat liver furnished metabolic products, [¹³C]-labeled pyruvate and lactate, originating from glycolysis. The levels of production and relative intensities of these metabolites were directly correlated with the induced glycolytic state (fasted versus fed groups). This work establishes hyperpolarized [¹³C]-Glyc as a novel agent for clinically relevant ¹³C MRS studies of energy metabolism and

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ASSOCIATED CONTENT

Supporting Information

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Experimental procedures and spectra of the described compounds (PDF)

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further provides opportunities for evaluating intracellular redox states in biochemical investigations.

Graphical Abstract

INTRODUCTION

The regulation of metabolic energy is fundamental to cell biology. Glycolysis, in particular, is a highly conserved pathway that furnishes ATP and NADH via the conversion of glucose to pyruvate.¹ Tissue-specific variations of glycolysis activity are found to balance the physiological needs of distinct cellular environments, and the pathway can be operative under both aerobic and anaerobic conditions.² Importantly, prolonged misregulation of glycolytic activity has become a metabolic signature of numerous pathological states such as various cancers, diabetes and heart diseases.³ The Warburg effect, for example, is a distinctive observation in cancer biology in which tumor cells exhibit higher levels of glycolysis even under aerobic conditions.⁴ In addition, pyruvate kinases (PK), which catalyze the final rate-limiting step of converting phosphoenolpyruvate (PEP) to pyruvate, display differential expression patterns in cancer cells,⁵ and the isozyme PKM2 has emerged as a novel target for cancer therapeutics using both small-molecule inhibitors and activators of the enzyme.⁶ Alterations in glycolytic metabolism have further been correlated with oxidative stress and changes in intracellular redox state.⁷

Given these well-established characteristics, diagnostic methods that adequately assess pathway activity *in vivo* are of critical importance.⁸ For example, positron emission tomography (PET) imaging with radiolabeled ^{18}F -fluorodeoxyglucose (FDG) has proven to be a valuable clinical method for both tumor detection and treatment response.⁹ This technique relies upon the high rate of glycolysis in many tumors, as FDG exhibits significant levels of uptake, phosphorylation and accumulation in certain cancerous tissues.¹⁰ However, FDG-PET is not always suitable for initial diagnosis of cancer¹¹ and only interrogates glucose transport and hexokinase activity (the first step of glycolysis), whereas many of the metabolic alterations in tumors and other pathologies are manifested at later stages of glycolytic metabolism.⁹⁻¹¹ Techniques that can evaluate flux throughout the glycolytic pathway *in vivo* remain limited.⁶

Hyperpolarized ^{13}C magnetic resonance spectroscopy (MRS) offers an innovative approach for real-time monitoring of metabolic pathways *in vivo*.¹² This technology has proven to evaluate physiological processes related to disease onset and progression including

metabolic changes associated with carcinogenesis.¹³ These methods rely upon the design of ^{13}C -labeled molecular probes that have several physicochemical properties: (1) effectively undergo nuclear spin-polarization, (2) target a specific biochemical pathway relevant to disease, and (3) furnish detectable concentrations of downstream metabolites during the experiment time frame (2–3 min).¹⁴ The development of hyperpolarized ^{13}C agents for the *in vivo* examination of glycolysis has received considerable attention. A 2014 report by Brindle and co-workers investigated hyperpolarized $[\text{U-}^2\text{H}, \text{U-}^{13}\text{C}]$ -glucose ($[\text{U-}^2\text{H}, \text{U-}^{13}\text{C}]\text{-Glu}$) in both T cell lymphoma (EL4) and Lewis lung carcinoma (LL2) mouse tumor models (Figure 1A).¹⁵ Glycolytic flux of $[\text{U-}^2\text{H}, \text{U-}^{13}\text{C}]\text{-Glu}$ was primarily observed, and ^{13}C -labeled lactate was identified as the major downstream metabolite. Other products of glycolysis (dihydroxyacetone phosphate and bicarbonate) and the pentose phosphate pathway (6-phosphogluconate) were detected at lower levels. Despite these advancements, $[\text{U-}^2\text{H}, \text{U-}^{13}\text{C}]\text{-Glu}$ displayed a short polarization lifetime ($T_1 \sim 9$ s),¹⁵ which reduced the clinical potential of the agent. In related studies of glycolytic metabolism, hyperpolarized $[\text{U-}^{13}\text{C}]\text{-dihydroxyacetone}$ ($[\text{U-}^{13}\text{C}]\text{-DHAc}$) was examined in several liver models (Figure 1A).¹⁶ In perfused liver, $[\text{U-}^{13}\text{C}]\text{-DHAc}$ was metabolized to furnish both glycolytic and gluconeogenic related intermediates.^{16a} Although the substrate possessed a $T_1 = 32$ s, the $[\text{U-}^{13}\text{C}]\text{-labeled}$ metabolic products have faster rates of polarization decay.¹⁷ In addition, only glycolysis-related PEP was detected in subsequent *in vivo* studies employing $[\text{U-}^{13}\text{C}]\text{-DHAc}$.^{16b} Other ^{13}C -labeled substrates in the glycolytic pathway have been polarized.¹⁸ However, only limited metabolic information has been obtained in subsequent *in vivo* animal studies. In addition, numerous methods utilizing hyperpolarized pyruvate have been described,^{12,13,19} but the detectable products provide an indirect measure of glycolytic state rather than a direct analysis of metabolic flux through the pathway. Given these issues, a clinically relevant hyperpolarized ^{13}C probe for the analysis of glycolytic metabolism has yet to be fully realized.

We have recently focused on the development of hyperpolarized ^{13}C substrates that can monitor alterations in energy production and cellular metabolism.²⁰ To address the challenges associated with examining glycolytic activity, our goal was to design an agent for dynamic nuclear polarization (DNP) that would effectively target this metabolic pathway. Glycerate-based substrates were an attractive scaffold for our initial investigations (Figure 1B), as glycerate has the potential to be metabolized via glycolysis.²¹ In addition, the C1-carbonyl carbon of glycerate could potentially be ^{13}C -labeled. Importantly, this labeling strategy would do the following: (1) obviate the need for ^1H decoupling, (2) provide suitable T_1 relaxation times for the substrate and resulting C1-labeled metabolites (Scheme 1), and (3) allow for longer experimentation times during hyperpolarized ^{13}C metabolic studies. These attributes would therefore address the primary issues found with hyperpolarized $[\text{U-}^2\text{H}, \text{U-}^{13}\text{C}]\text{-Glu}$ and $[\text{U-}^{13}\text{C}]\text{-DHAc}$. To this end, this report describes the synthesis, development, and *in vivo* application of sodium $[\text{U-}^{13}\text{C}]\text{-glycerate}$ ($[\text{U-}^{13}\text{C}]\text{-Glyc}$). Hyperpolarized $[\text{U-}^{13}\text{C}]\text{-Glyc}$, which displayed a high level of polarization with a long spin–lattice relaxation time, successfully furnished metabolic products of glycolysis. These studies enhance the clinical applications of hyperpolarized ^{13}C MR by providing a new potential diagnostic tool for assessing alterations in metabolic energy states *in vivo*.

MATERIALS AND METHODS

Synthesis of [^{13}C]-Glyc

All synthetic procedures and compound characterization data are detailed in the [Supporting Information](#).

Dynamic Nuclear Polarization of [^{13}C]-Glyc

The samples to be polarized consisted of a mixture of [^{13}C]-Glyc in 3.0 M glycerol/water (3/2, v/v) solution containing 15 mM trityl radical OX063 (Oxford Instruments Molecular Biotools, Oxford, UK). The samples were polarized via dynamic nuclear polarization using either a SPINLab (General Electric, Niskayuna, New York, USA) or HyperSense system (Oxford Instruments Molecular Biotools, Oxford, UK). The polarized samples were dissolved in a solution of 40 mM Tris buffer, 50 mM NaCl, and 0.1 g/L EDTA- Na_2 , leading to an 80 mM solution of the hyperpolarized substrate with a pH of approximately 7.5. The resulting buffered hyperpolarized [^{13}C]-Glyc solution was used directly in T_1 measurements at 3 T.

In Vivo Experiments

Healthy male Wistar rats (fed: 368 ± 86 g body weight, fasted (16–20 h): 374 ± 81 g body weight, $n = 3$ per group) were injected with 2.6–3.2 mL of the hyperpolarized solution (target dose = 1 mmol/kg body weight) through a tail vein catheter at a rate of approximately 0.25 mL/s. The time from dissolution to start of injection was approximately 20 s. The rats were anesthetized initially with 2.5% isoflurane in oxygen (1.5 L/min) for tail vein catheterization. Respiration, rectal temperature, heart rate, and oxygen saturation were monitored throughout the experiments with temperature regulated using a warm water blanket placed underneath the animals. All animal procedures were approved by the Stanford University Institutional Animal Care and Use Committee. All experiments were performed on a clinical 3T MR scanner (GE Healthcare, Waukesha, WI), using a custom-built ^{13}C transmit/receive surface coil (diameter = 28 mm) placed over the liver with rat supine. A quadrature volume rat ^1H coil (diameter = 70 mm) was used for anatomical localization and to confirm the position of the ^{13}C coil with respect to the liver. Single-shot fast spin-echo (FSE) ^1H MR images in the axial, sagittal, and coronal planes with nominal in-plane resolution of 0.47 and 2 mm slice thickness were acquired as anatomical references for prescribing the ^{13}C MRS experiments. A nonselective pulse-and-acquire sequence with an excitation flip angle of 10° , spectral width of 5 kHz, and 2048 points was used to acquire ^{13}C spectra from the liver every 3 s over a 4 min period starting at the same time as the [^{13}C]-Glyc injection.

RESULTS AND DISCUSSION

Synthesis and Dynamic Nuclear Polarization of [^{13}C]-Glyc

A practical synthesis of [^{13}C]-Glyc was initially accomplished via a four-step synthetic route (Scheme 2).²² In this sequence, commercially available [1- ^{13}C]-acetic acid (\$7.33/mmol, Cambridge Isotopes) underwent α -halogenation under acidic conditions with Br_2 followed by esterification with benzyl alcohol to provide an 85% yield of the benzyl ester, **1**. Then **1**

was reacted with PPh_3 and aqueous NaOH to furnish the corresponding ylide, which was subsequently treated with formaldehyde to provide ^{13}C -labeled benzyl acrylate **2**. Dihydroxylation of **2** with $\text{ad mix-}\alpha$ successfully afforded **3** in a 66% yield over two steps. To complete the synthesis of ^{13}C -Glyc, **3** was hydrogenated with palladium on carbon to quantitatively remove the benzyl protecting group. Exposure of the resulting reaction mixture to NaHCO_3 provided ^{13}C -Glyc, which displayed a single downfield ^{13}C signal at 178.9 ppm in the ^{13}C NMR spectrum (Figure 2A). Overall, this synthetic route provided ^{13}C -Glyc in a 56% yield over four steps. In addition, the process could be performed on a one-gram scale to directly furnish 1.18 g of ^{13}C -Glyc for hyperpolarized studies.

Formulation and DNP studies were conducted with ^{13}C -Glyc to establish conditions for hyperpolarized ^{13}C MRS experiments. Although ^{13}C -Glyc was highly soluble in water (1.0 g/mL),²³ the DNP process required the sample to form a glass upon freezing to <2 K. Therefore, a 3.0 M ^{13}C -Glyc sample was prepared by dissolving the ^{13}C -labeled substrate in a mixture of glycerol/water (3/2, v/v). The sample also required the addition of a free radical OX063 (15 mM). At low temperatures, this one-electron species has a high level of polarization, which is then transferred to ^{13}C nuclear spins upon microwave irradiation. The DNP process thus results in a substantial enhancement of ^{13}C NMR signal. ^{13}C -Glyc samples were polarized via DNP using either a SPINLab or HyperSense system. Figure 2B displays a representative solid-state polarization buildup. Upon dissolution, $19.7\% \pm 0.9\%$ ($n = 3$ measurements, mean $\pm$ STE) liquid-state polarization was detected at the scanner (~ 35 s after dissolution), which corresponded to a $\sim 70\,000$ -fold signal enhancement (SPIN-Lab). The T_1 of the ^{13}C -labeled carbonyl was determined to be 59.9 ± 3.0 s at 3 T in the hyperpolarized solution (Figure 2C), which importantly represented a significant increase in relaxation time relative to previous hyperpolarized agents, $^{2}\text{H}, ^{13}\text{C}$ -Glu and ^{13}C -DHAc. In addition, since ^{13}C -Glyc was labeled at the C1 position, the resulting C1-labeled metabolic products in hyperpolarized ^{13}C MRS studies would be expected to also display suitably long relaxation times.

Hyperpolarized ^{13}C MRS Studies

The validation of hyperpolarized ^{13}C -Glyc would provide new clinical opportunities. For example, a radioactive tracer has recently been developed to query the status of PKM2 expression in vivo using PET imaging.²⁴ This imaging agent would be ideally suited as a companion diagnostic to enroll patients onto any PKM2-targeted therapies, and hyperpolarized ^{13}C -Glyc could then be used to assay PKM2 activity and monitor effective therapy.

To evaluate the response of ^{13}C -Glyc under various physiological conditions, our approach was to monitor the in vivo metabolism of ^{13}C -Glyc under distinct glycolytic states in the liver, as this organ plays a key role in regulating energy metabolism.²⁵ In the fed state, glucose is metabolized via glycolysis to generate pyruvate, which further generates ATP through the TCA cycle and oxidative phosphorylation in the mitochondria. However, during prolonged fasting, glycolysis activity is diminished, and hepatocytes synthesize glucose from precursors (e.g., lactate, pyruvate, glycerol, and amino acids) via gluconeogenesis. The transition from a glycolytic to a gluconeogenic state in the liver is paralleled by an

increasingly reduced cytosolic compartment.²⁶ In vivo hyperpolarized ^{13}C MRS studies with ^{13}C -Glyc were thus conducted in rat liver as this model allowed for readily inducing both glycolytic (fed) and nonglycolytic (fasted) states.

Healthy male Wistar rats were employed for the investigation of hyperpolarized ^{13}C -Glyc, and all experiments were performed on a clinical 3 T MR scanner. No observable toxicity (pulse or respiration) was detected upon intravenous administration of a Tris buffered solution containing up to 80 mM ^{13}C -Glyc. Figure 3 displays representative spectra (3A and 3B) and time courses (3C and 3D) obtained after administration of hyperpolarized ^{13}C -Glyc to fasted and fed animals. In all experiments, the substrate was observed at 178.9 ppm, and two major metabolic products were found: $[1-^{13}\text{C}]$ -pyruvate (^{13}C -Pyr, 172.1 ppm) and $[1-^{13}\text{C}]$ -lactate (^{13}C -Lac, 185.1 ppm). $[1-^{13}\text{C}]$ -bicarbonate (161.4 ppm) was also detected at lower levels in two of the fed animals. In all cases, the metabolic products were observed over the first 100–120 s of the experiment with the maximum signal detected after 20–30 s. Lipid contribution to the ^{13}C -Pyr signal was minimal, as determined by control scans prior to substrate injection.²⁷ ^{13}C -labeled alanine likely could not be distinguished because of spectral overlap with ^{13}C -Glyc. In addition, phosphoglycerate intermediates are known to have similar chemical shifts as the substrate.²⁸ Although the precise method of entry into glycolysis cannot be determined by these initial studies alone, these results were consistent with glycolytic flux of ^{13}C -Glyc in the liver, demonstrating the hyperpolarized ^{13}C MR spectroscopy with the agent successfully targeted the metabolic pathway in vivo.

Figure 4 summarizes the metabolic differences obtained from the fasted versus fed groups in ^{13}C MRS experiments employing hyperpolarized ^{13}C -Glyc. The contribution of the metabolites was determined by examining the area under the curve for the time courses (mean $\pm$ STE). The total signal from downstream products provided an estimation of flux in both states (Figure 4A). Glycolytic activity was reduced by 34% in the fasted versus fed groups (1.18 ± 0.25 versus 0.78 ± 0.16 , respectively) at a $P < 0.05$ confidence level. These results with hyperpolarized ^{13}C -Glyc were consistent with the gluconeogenic state, where carbon precursors are consumed for de novo glucose synthesis. When the individual metabolite levels were examined as a fraction of total ^{13}C signal (Figure 4B), ^{13}C -Lac displayed a significant decrease in the fasted versus fed groups ($P < 0.05$). Previous studies of liver metabolism have also found similar changes in lactate pool sizes.²⁹ However, ^{13}C -Pyr levels were detected in a comparable range in both groups. Previous reports have observed a chemical shift of $[1-^{13}\text{C}]$ -PEP (^{13}C -PEP) in a similar region as ^{13}C -Pyr with a 1.7 ppm difference in the respective signals.³⁰ In our study at 3 T, spectral overlap of these metabolites cannot be discounted, which could result in potential contribution of ^{13}C -PEP to the ^{13}C -Pyr signal. Since the interconversion between pyruvate and lactate catalyzed by lactate dehydrogenase is generally in a fast chemical equilibrium, the lactate/pyruvate ratio has traditionally been employed in the evaluation of intracellular redox states.³¹ These biochemical measurements have recently been performed in vitro via hyperpolarized ^{13}C MRS using $[2\text{H},^{13}\text{C}]$ -Glu, which provided analyses of two cancer cell lines.³² In our in vivo experiments with hyperpolarized ^{13}C -Glyc, ^{13}C -Lac/ ^{13}C -Pyr ratios were determined to be 8.89 ± 3.15 for the fed group (Figure 4C). Under normal physiological conditions, the cytosolic lactate/pyruvate ratio is maintained at $\sim 10/1$,^{26,33} correlating strongly with the

hyperpolarized [^{13}C]-Glyc analysis. However, since no detectable difference in pyruvate to total carbon area was observed between the groups, a statistically significant change in the [^{13}C]-Lac/[^{13}C]-Pyr ratio was not detected in the fasted relative to the fed state. In addition, because of the lack of change in pyruvate fraction in the fasted group, the [^{13}C]-Lac/[^{13}C]-Pyr ratio was found to be lower in this state (5.58 ± 1.25). Similar trends in lactate/pyruvate measurements have been observed in studies of liver metabolism.^{16a,34} However, under different experimental conditions, previous analyses²⁶ have indicated a more reducing environment in excised rat liver tissues after prolonged fasting but with relatively large levels of uncertainty in the lactate/pyruvate ratio after 48 h starvation. Even though our data was indicative of a similar pyruvate fraction for all animals, it is difficult to be certain that fasting had no effect on pyruvate levels—and thereby affecting the estimated [^{13}C]-Lac/[^{13}C]-Pyr ratio—in our study with hyperpolarized [^{13}C]-Glyc. The presence of an overlapping signal from [^{13}C]-PEP may further complicate the measurements, as this metabolite has been shown to vary in concentration under fasted and fed states in the liver.³⁵ In addition, previous hyperpolarized ^{13}C studies of liver metabolism have uncovered that pyruvate is metabolized by divergent pathways in the fed versus fasted states,³⁶ which may affect the metabolite pool size and the rate of pyruvate metabolism and further influence the quantification. To improve cytosol redox measurements, future methodological work will focus on better quantification of [^{13}C]-Pyr levels through increasing signal-to-noise and distinguishing any contribution of [^{13}C]-PEP. Importantly though, hyperpolarized [^{13}C]-Glyc was able to provide [^{13}C]-Lac/[^{13}C]-Pyr in agreement with normal physiological conditions. In addition, the agent successfully distinguished the glycolytic states in the liver with respect to overall flux through the pathway and the levels of lactate production.

CONCLUSION

In summary, we have described the application of [^{13}C]-Glyc as a novel probe for assessing glycolysis in hyperpolarized ^{13}C spectroscopic studies. The ^{13}C -labeled substrate was synthesized in high yield from inexpensive, commercially available starting materials. Upon formulation of [^{13}C]-Glyc for DNP studies, the substrate displayed high polarization levels with a long spin–lattice relaxation time, obviating the clinical challenges associated with related agents. Importantly, hyperpolarized [^{13}C]-Glyc successfully targeted the glycolytic pathway in vivo, and metabolic products, [^{13}C]-Pyr and [^{13}C]-Lac, were observed. Metabolite levels were correlated with the glycolytic state in rat liver, which establish hyperpolarized [^{13}C]-Glyc as a promising diagnostic probe for analyzing glycolysis. Future work will focus on implementing [^{13}C]-Glyc in PKM2-related cancer models and extending these spectroscopic methods toward the evaluation of intracellular redox environments.

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17. Representative examples of T_1 relaxation times for $[1-^{13}\text{C}]$ - vs $[1-^{13}\text{C}]$ -labeled metabolic products at 3 T (refs 14b and 14c): $[1-^{13}\text{C}]$ -pyruvate = 60 s and $[2-^{13}\text{C}]$ -pyruvate = 44 s; $[1-^{13}\text{C}]$ -lactate = 46 s and $[2-^{13}\text{C}]$ -lactate = 6 s.
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Figure 1. Chemical probes for evaluating glycolysis metabolism with hyperpolarized ^{13}C MRS. (A) Previous work with $[U\text{-}^2\text{H}, U\text{-}^{13}\text{C}]$ -glucose (ref 15) and $[2\text{-}^{13}\text{C}]$ dihydroxyacetone (ref 16) and (B) our work with sodium $[1\text{-}^{13}\text{C}]$ glycerate.

Figure 2.

(A) High-resolution NMR spectrum of ^{13}C -Glyc (carbonyl region). (B) Polarization buildup curves (3.35 T, 1.4 K) of ^{13}C -Glyc in glycerol–water doped with 15 mM OX063 taken at $P(+) = 94.125$ GHz (HyperSense). (C) Liquid-state T_1 decay (3 T, 298 K) of ^{13}C -Glyc samples with 15 mM OX063 ($T_1 = 59.9 \pm 3.0$ s).

Figure 3.

Representative time-averaged spectra obtained from liver beginning at the administration of 80 mM hyperpolarized [¹³C]-Glyc for (A) fasted and (B) fed groups. Representative time course obtained from liver for (C) fasted and (D) fed groups.

Figure 4.

Contribution of the individual metabolites was determined by examining the area under the curve for the time courses. (A) Ratio of total detected metabolic products ($[^{13}\text{C}]\text{-Lac} + [^{13}\text{C}]\text{-Pyr}$) to $[^{13}\text{C}]\text{-Glyc}$ for fasted and fed groups (mean $\pm$ STE). (B) Fractional contributions of individual metabolites $[^{13}\text{C}]\text{-Lac}$ and $[^{13}\text{C}]\text{-Pyr}$ to total ^{13}C signal for fasted and fed groups. (C) $[^{13}\text{C}]\text{-Lac}$ to $[^{13}\text{C}]\text{-Pyr}$ ratios for fasted and fed groups. (+: $P < 0.05$).

Scheme 1. Potential Pathway for the Metabolism of [^{13}C]-Glyc

Scheme 2. Synthesis of $[^{13}\text{C}]$ -Glyc^a

^aReaction conditions: (a) TFA, DMAP, Br_2 , 60 °C; benzyl alcohol, rt to 60 °C, 85% yield.

(b) PPh_3 , toluene; NaOH (aq) CH_2Cl_2 ; CH_2O , CH_2Cl_2 . (c) Ad mix alpha, $^t\text{BuOH}$, H_2O , 4 °C, 66% yield (2 steps). (d) Pd/C , H_2 , $\text{THF/H}_2\text{O}$; NaHCO_3 (aq), 99% yield. (Red circle = ^{13}C).