A Plausible Metal-Free Ancestral Analogue of the Krebs Cycle Composed Entirely of α-Ketoacids.

R. Trent Stubbs[1,3], **Mahipal Yadav**[2,3], **Ramanarayanan Krishnamurthy**[*,2,3], **Greg Springsteen**[*,1,3]

[1]Department of Chemistry, Furman University, 3300 Poinsett Hwy, Greenville, SC 29613, USA

[2]Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

[3]NSF-NASA Center for Chemical Evolution, Atlanta, GA 30332, USA

Abstract

Efforts to decipher the prebiotic roots of metabolic pathways have focused on recapitulating modern biological transformations, with metals typically serving in place of cofactors and enzymes. Here, we show that the reaction of glyoxylate with pyruvate under mild aqueous conditions produces a series of α-ketoacid analogs of the reductive citric acid cycle, without the need for metals or enzyme catalysts. The transformations proceed in the same sequence as the reverse Krebs cycle, resembling a proto-metabolic pathway, with glyoxylate acting as both the carbon source and reducing agent. Additionally, the α-ketoacid analogs provide a natural route for the synthesis of amino acids by transamination with glycine, paralleling the extant metabolic mechanisms, and obviating the need for metal-catalyzed abiotic reductive aminations. This emerging sequence of prebiotic reactions could have set the stage for the advent of increasingly sophisticated pathways operating under catalytic control.

The discovery of abiotic chemical pathways that resemble the extant citric acid (Krebs, tricarboxylic acid (TCA)) and glyoxylate cycles might help explain both the ubiquity of these pathways in modern biology and the centrality of their intermediates in metabolism. Genomic and metabolic analyses suggest that a precursor to the modern TCA cycle was active near the origins of extant metabolism, likely in an anabolic role.[1–6] These ancestral pathways likely did not complete a metabolic cycle, but rather simultaneously employed parts of both the oxidative (o) and reductive (r) TCA cycles to transform pyruvate, a prebiotically plausible α-ketoacid,[7–9] into larger organic building blocks including α-

ketoglutarate and succinate.[10] Together with acetyl-CoA and oxaloacetate, these compounds are core biosynthetic species that feed much of modern metabolism (outer cycles, Fig. 1).[11] The glyoxylate cycle, a variant of the TCA that bypasses decarboxylation steps, has also been proposed as a potential evolutionary predecessor (green pathway, Fig. 1).[12]

There have been many efforts to recapitulate the reactions and transformations of the TCA cycle and almost all of them rely on chemistries that are metal-mediated.[13–16] This is a consequence of a focus on directly connecting modern enzyme-enabled TCA pathways with transition metal-based autotrophic origins in the belief that metals (in minerals) present in a non-enzymatic environment served as catalysts and were replaced later by protein enzymes.[6,17–21] However, in spite of many efforts,[22] bridging the gap from plausible prebiotic chemistry has been a tremendous challenge, and no anabolically productive (C-C bond-forming) step of either cycle appears to progress appreciably without enzyme catalysis or extreme environments, even when supplied with appropriate cofactors.[23–26] Therefore, skepticism about the existence of abiotic precursors to the TCA and glyoxylate cycles has been expressed in the literature both in regards to the potential rates of such transformations without enzyme catalysis,[24] and the improbability of identifying common conditions that support all of the reaction types.[27] This reasonable pessimism stems from the chemical complexity of the TCA cycle, which utilizes multiple carboxylic acid intermediates that are substantially unreactive without highly competent enzymes and multiple specialized cofactors.

Rather than a top-down approach that focuses on the intermediates of TCA cycle, we have employed a strategy of identifying the chemistries that underlie the TCA cycle and applying them to molecules that could have existed on early earth. Using this approach, we previously demonstrated that a mixture of pyruvate and glyoxylate produces an efficient reaction sequence resembling a proto-metabolic cycle, where malonate is regenerated (through oxaloacetate) via the consecutive addition of glyoxylate and hydrogen peroxide (H_2O_2) in a single pot.[28] In this previously-reported work, malate and oxaloacetate were the only TCA intermediates that were produced. Extending this research, recent work has demonstrated that the pyruvate and glyoxylate reaction, in the presence of transition metal ions, generates a heterogeneous mixture of TCA intermediates at mildly acidic pH (~4.5 to 5.5) and elevated temperature (70 °C).[29] However, the metal catalysis also leads to the breakdown of these intermediates creating a complex mixture of products, which decompose over time resulting in low yields. Thus, with no clear potential for the sustainable generation of these metabolites, a viable abiotic evolutionary predecessor to the TCA cycle remains elusive.

The primary issue with replicating the chemistry of the modern TCA cycle in an abiotic environment is that all of the polycarboxylate intermediates that lack α-ketoacids are pi(π)-electron rich and, therefore, weakly acidic at the α-carbon. This disfavors deprotonation reactions to enolic intermediates, and consequently, proficient catalysts (or harsh conditions) are required. However, simpler but related pathways —with substantial abiotic reaction rates — may have preceded these canonical routes, providing a foundation for the subsequent evolution of the modern cycles.[1,30] Absent competent catalysts, the chemical species within these progenitor pathways would have required reactive functional groups whose generation,

persistence and transformation was consistent with plausible prebiotic chemistry. A clue to this functional group enabled reactivity might be found within the modern TCA cycle itself, which contains two intermediates with an α-ketoacid functional group ($RC(O)CO_2^-$), oxaloacetate and α-ketoglutarate (Fig. 1). The α-carbon of the α-ketoacid group has greatly increased acidity versus the α-carbon of a carboxylate (by $\sim 10^6$ for acetate versus pyruvate).[31,32] The α-ketoacids also have relatively higher enol content, attributed to: (a) the destabilization of the keto isomer by the adjacent carboxylate group and (b) the stabilization of the enol form by the carboxylate group.[31,33] These properties, in combination with an electron-deficient carbonyl group, enables the α-ketoacids to behave as nucleophiles as well as electrophiles, capable of reacting under mild aqueous conditions.[28,32]

This observation suggests that a primordial pathway comprised of only α-ketoacids might operate without the need for catalysts or extreme environments. This chemistry may have then served as a foundational pathway that subsequently evolved (via metal and peptide catalyzed pathways) into the extant TCA cycle, which now operates under exclusive control of sophisticated catalysts and cofactors. Herein, we report an analog of the r-TCA cycle that features only the corresponding α-ketoacid equivalents of modern intermediates. The pathway relies on only two reactants, pyruvate (I) and glyoxylate (II), and requires no transition metals or harsh conditions to generate the α-ketoacid analogs of malate, fumarate, succinate, α-ketoglutarate, isocitrate and aconitate in the same sequence as the r-TCA cycle. Importantly, glyoxylate itself acts as both the carbon source and the reductant, which dramatically simplifies the chemistry and produces an uncluttered reaction sequence in comparison to the modern biochemistry. All of the transformations along the α-ketoacid pathway progress over a range of pH ($\approx$ 7–8.2) in water, without the external addition of any oxidants or reductants.

Results:

We began with investigating the reaction of pyruvate (I) and glyoxylate (II) in the absence of any metal or enzyme catalyst. We observed that a reaction at pH 7 in 0.5 M phosphate buffer heated to 50 °C gave rise to, in a single pot, the corresponding five α-ketoacid analogs (III-VII) as the major products in a combined 57% yield, within 21 h, as documented by ^{1}H-NMR (Fig. 2). In the first step, an aldol addition of glyoxylate to pyruvate produced the α-ketoacid variant of malate (III, maloyl formate or 4-hydroxy-2-ketoglutarate, HKG) in 39% yield (Fig. 2d). Subsequent dehydration yielded fumaroyl formate (IV, the α-ketoacid equivalent of fumarate). An irreversible reduction of fumaroyl formate then generated the canonical TCA intermediate α-ketoglutarate (V), followed by an additional aldol addition with glyoxylate (II) to produce isocitroyl formate (VI, the α-ketoacid equivalent of isocitrate). The subsequent dehydration gave rise to aconitoyl formate (VII, the α-ketoacid equivalent of aconitate) for a total yield of 57% of the reaction intermediates in a single-pot reaction sequence.

In the modern r-TCA cycle, α-ketoglutarate is generated by the reductive carboxylation of succinate, and the catalytic complexity of this transformation is a significant hurdle to abiotic r-TCA cycle models.[23,34] In the analog pathway demonstrated here, the reduction of fumaroyl formate to α-ketoglutarate is mediated by glyoxylate and requires no transition

metals or other conventional reductants. This reaction is glyoxylate dependent (Fig. 3) and likely proceeds via a cross-Cannizzaro type mechanism by hydride transfer from the hydrated glyoxylate to the conjugated carbon-carbon double bond of fumaroyl formate (Fig. 3a). The over production of oxalate versus glycolate, as confirmed by ^{13}C-NMR analysis (Supplementary Fig. 1), is consistent with this hypothesis. An alternative pathway for the reduction, which contributes equally, was also found to be operative in which a second aldol addition of glyoxylate to maloyl formate produced a double adduct (Di-HKG, Supplementary Figs. 1, 2), which then undergoes dehydration, decarboxylation, and an irreversible retro-Claisen fragmentation to form, again, α-ketoglutarate. The formate byproduct generated by this transformation was also observed by ^{13}C-NMR (Supplementary Fig. 1). Multiple routes to produce the same intermediate (α-ketoglutarate) highlights the opportunities and robustness of this reaction network. Additionally, the reduction to α-ketoglutarate is irreversible, providing a ratcheting mechanism to capture the anabolic potential of this pathway. The formation of α-ketoglutarate from pyruvate and glyoxylate proceeds slowly in water (Supplementary Fig. 6) and faster in the presence of multiple buffers including phosphate (pH ≈ 7), cacodylate (pH ≈ 7), and bicarbonate (pH ≈ 8.2); the rate enhancement observed on increasing ion (buffer) concentration likely indicates the buffers are acting as general acid-base catalysts (Supplementary Fig. 16).

When the reaction sequence with glyoxylate was started from α-ketoglutarate (in place of pyruvate), the formation of the isocitrate and aconitate analogs was even more clearly observed. The aldol addition of glyoxylate (II) to α-ketoglutarate (V) yielded 36% of isocitroyl formate (VI) in a 2:1 mixture of the erythro and threo diastereomers (Fig. 2c). The dehydration to aconitoyl formate (VII) proceeded in 21% yield. These addition and dehydration steps are in an active equilibrium, which was confirmed by the production of ^{13}C-γ,δ-labeled α-ketoglutarate from the reaction of unlabeled α-ketoglutarate and ^{13}C2-labeled glyoxylate, demonstrating the reversibility of the reaction to aconitoyl formate (Supplementary Fig. 3).

The sequence of reactions starting from pyruvate and glyoxylate, as observed by NMR, is also supported by a time-course HPLC analysis of reaction aliquots that were quantified by comparison to the UV-active canonical TCA species (Fig. 4a). The production of fumaroyl formate preceded the appearance of aconitoyl formate as the reaction progressed along the analog pathway. The reaction pathway could also be started from oxaloacetate, which produced a similar product distribution as from pyruvate (Fig. 1, Supplementary Fig. 4).[35,36] Additionally, the dimerization of oxaloacetate (VIII)[28] produced citroyl formate (IX),[37] providing a route to the α-ketoacid analog of the last major constituent of the r-TCA cycle (Fig. 1, Supplementary Fig. 5).[37] Thus, the complete set of α-ketoacid analogs of each of the r-TCA cycle intermediates can be accessed starting only from the α-ketoacids: glyoxylate, pyruvate, and oxaloacetate.

The oxidative decarboxylation of α-ketoacids to give the corresponding carboxylic acids is well documented.[37] The addition of H_2O_2 to the reaction mixture generates the corresponding carboxylic acids, identical to the canonical TCA cycle intermediates, within 30 minutes in quantitative yield (Fig. 4b, c). Although the oxidative decarboxylation of α-ketoacids to the canonical r-TCA intermediates in a prebiotic context might be seen as a

desired outcome, this transformation may have been disadvantageous prior to the emergence of competent catalysts, due to the lack of reactivity of the resulting carboxylic acids.

The increased electrophilicity of the carbonyl group of α-ketoacids, as compared to carboxylic acids, also provides a prebiotically plausible route for the generation of α-amino acids that parallels extant biology and eliminates the need for metal-mediated reductive amination.[21] Under acid-catalyzed conditions, the α-ketoacid intermediates are converted into α-amino acids by a transamination mechanism,[38] using glycine as a source of the amino group. The addition of α-ketoglutarate to glycine in a pH 5 aqueous solution containing potassium aluminum sulfate $(AlK(SO_4)_2$, alunite)[39–41] generated glutamate in a 44% yield (Fig. 5a) after 4 h at 80 °C. Under similar conditions, pyruvate was converted into alanine in 19% yield after 24 h (Fig. 5b). These transformations allow for the synthesis of a diversity of amino acids solely from the simplest amino acid, glycine, which has been shown to be prebiotically available via multiple pathways including meteoritic sources.[42,43] Glycine is also advantageous in that the transamination by-product, glyoxylate, can be recycled directly back into the α-ketoacid-analog pathway.

The transition-metal free reaction of pyruvate and glyoxylate, and the reduction to form α-ketoglutarate documented in our work contrasts with a recent report,[29] implicating that Fe^{2+} mediates this transformation. When the glyoxylate and pyruvate reaction was carried out under the conditions described,[29] unbuffered at pH 4.5 and 70 °C, (but in the absence of the iron), we observed that the reaction proceeds and, indeed, produces α-ketoglutarate as seen by ^1H-NMR (Supplementary Fig. 6) — all in the absence of Fe^{2+}. These observations strongly suggest that glyoxylate could be the actual reductant, and that Fe^{2+} may not be necessary. Additionally, although Fe^{3+}, produced by the in situ oxidation of Fe^{2+}, induces the oxidative decarboxylation of α-ketoacids to form the canonical intermediates,[29] it is also destructive to product accumulation[44,45] — as evidenced by the continuous degradation of carbon-based material.[29] Together, these data suggest that not only are transition metals, such as Fe^{2+}, not required for a reductive proto-metabolism, they may actually be deleterious if not sequestered as they are in extant biology.[46]

Discussion:

The demonstration here that a small organic and plausible prebiotic building block, glyoxylate, can serve as both a carbon source and a reductant, while under conditions that are not degradative to the reactants or products, strongly supports the hypothesis that an early proto-metabolism with anabolic potential can emerge under mild aqueous, metal-free conditions. Additional remnants of the α-ketoacid r-TCA analog chemistry demonstrated in this work might be found in the glyoxylate shunt in extant biology (Fig. 1), which uses glyoxylate as an anabolic electrophile to generate TCA cycle intermediates. Both the analog pathway shown here and the glyoxylate shunt, which has been suggested as a TCA precursor,[12] avoid any steps involving the production or consumption of CO_2, suggesting that the mechanistic complexity of CO_2 processing steps might have required the catalytic sophistication of an evolved metabolism.[44] This analog pathway and the modern TCA cycle also rely on pyruvate for the generation of intermediates, suggesting its plausibility as an evolutionary remnant. Glyoxylate[47] and pyruvate[7–9] are also the two smallest α-ketoacids,

with just two and three carbons respectively, and may have been prebiotically available for the origins of proto-metabolism.

While we recognize it is potentially misleading to make direct connections between prebiotic chemistry and extant biology,[44,48] it seems possible that there could be a natural evolutionary transition forward from this α-ketoacid-based chemistry. For example, the only remaining α-ketoacids in the extant cycle, oxaloacetate and α-ketoglutarate, are the sole TCA-cycle sources of canonical amino acids, raising questions as to why they have been preserved. In modern metabolism, α-ketoglutarate is transaminated into glutamate (which is then transformed into glutamine, proline, and arginine), and oxaloacetate is transaminated into aspartate (which is then transformed into asparagine, methionine, threonine, and lysine).[11] Such a centrality of these two canonical amino acids might presuppose that they conferred some selective advantage to an evolving metabolism, and that the persistence of their source α-ketoacids might have been a natural consequence of this functionality. From a purely structural-reactivity perspective, glyoxylate, pyruvate, oxaloacetate and α-ketoglutarate are also the only species that are resistant to side reactions that prevent, or compete with, a transamination to form amino acids. All the others are either sterically hindered at the α-carbon atom or have unsaturation or functional groups that may impact their reactivity (Supplementary Fig. 9). As the proto-metabolism evolved from the α-ketoacid analog pathway, it may have been advantageous to replace the remaining α-ketoacids with their corresponding polycarboxylates as catalytic control became possible, potentially to increase regulatory control due to the inherent stability of these extant metabolites.

The existence of a reaction pathway that progresses under prebiotically plausible conditions through the exact α-ketoacid equivalents of the TCA cycle, while circumventing a mechanistically difficult reductive carboxylation step, suggests that the polycarboxylate chemistry of the modern cycle may have been an invention of subsequent evolution. Similarly, the reactivity of the α-ketoacid-carbonyl group allows for a simpler transamination reaction for the production of amino acids without the need for reducing metals. These observations suggest that close analogs of modern biochemical pathways might have proceeded under plausible abiotic conditions and argue against the widely held perception that an external inorganic reductant is necessary for a primordial metabolic network of reactions. Furthermore, the chemistry of α-ketoacids have the potential to enable a diverse suite of transformations critical to an emerging proto-metabolism, including peptide coupling (through decarboxylative amidation) and thioester synthesis.[49,50]

Methods:

All reactions were run under an atmosphere of N_2. All reagents were used as purchased from their chemical manufacturer: sodium pyruvate (99%, ReagentPlus, Sigma-Aldrich), α-ketoglutaric acid disodium salt dihydrate (98%, Aldrich Chemistry), oxaloacetic acid (97%, Sigma-Aldrich), 50 wt% in H_2O glyoxylic acid solution (Sigma-Aldrich), aluminum potassium sulfate dodecahydrate (BioReagent, Sigma Life Science), (DL)-4-hydroxy-2-ketoglutaric acid lithium salt (90%, Sigma Life Science, maloyl formate), $(1,2-^{13}C_2)$-glyoxylic acid monohydrate (99%, Cambridge Isotope Laboratories, Inc.) and 30 wt% in

H_2O hydrogen peroxide solution (contains inhibitor, assayed 29.0 to 32.0%, Sigma-Aldrich 216763). ^{1}H-NMR spectra were collected using a Varian 400-MR 400 MHz high-field superconducting NMR spectrometer with chemical shifts (δ) reported in ppm. ^{13}C-NMR spectra were recorded at 298K using a Bruker AV-600 (151 MHz for ^{13}C) equipped with a 5 mm DCH cryoprobe. NMR samples were prepared by adding 100 μL of the crude reaction mixture to 600 μL of D_2O containing a t-butanol internal standard. HPLC chromatograms were collected using a Waters Alliance e2695 Separations Module coupled to a Waters 2998 Photodiode Array Detector. HPLC samples were prepared by adding 300 μL of the crude reaction mixture to 1.55 mL of 0.1% formic acid in water. Samples were eluted with an isocratic mobile phase of 0.1% formic acid in water at 0.75 mL/min for 20 min on a Synergi 4u Polar-RP 80A (250 × 4.6 mm) column and visualized at by UV absorbance at 210 nm. pH values of solutions were measured using a Fisherbrand Accumet XL150 pH meter with an Orion 9110DJWP Double Junction pH Electrode.

Conversion of pyruvate to α-ketoacid analogs (III, IV, V, VI, VII) followed by oxidation to TCA cycle intermediates:

110 mg of sodium pyruvate (1.00 mmol, 200 mM) was dissolved in 4.2 mL of a 0.50 M aq. pH 7 sodium phosphate buffer in a 20-mL vial. Then, 331 μL (222 mg) of 50 wt% glyoxylic acid in H_2O (3.0 equiv.) was added. The reaction pH was adjusted to 7.0 by adding 4.0 M aq. NaOH dropwise. The reaction was stirred at 50 °C for 21 h to yield α-ketoacid intermediates (39% maloyl formate, 6% fumaroyl formate, 5% α-ketoglutarate, 5% isocitroyl formate, 2% aconitoyl formate, and 1% pyruvate remaining). Then, 511 μL (170 mg) of 30 wt% hydrogen peroxide in H_2O (5.0 equiv.) was added, and the resulting solution was stirred at rt for 30 min to yield TCA cycle intermediates (39% malate, 6% fumarate, 5% succinate, 5% isocitrate, and 2% aconitate).

Conversion of α-ketoglutarate to α-ketoacid analogs (VI and VII) followed by oxidation to TCA cycle intermediates:

452 mg of α-ketoglutaric acid disodium salt dihydrate (2.00 mmol, 200 mM) was dissolved in 8.8 mL of a 0.50 M aq. pH 7 sodium phosphate buffer in a 20-mL vial. Then, 221 μL (148 mg) of 50 wt% glyoxylic acid in H_2O (1.0 equiv.) was added. The reaction pH was adjusted to 7.0 by adding 4.0 M aq. NaOH dropwise. The reaction was stirred at 50 °C for 21 h to yield α-ketoacid intermediates (isocitroyl formate: 36%, aconitoyl formate: 21%, α-ketoglutarate remaining: 37%). Then, 1021 μL (340 mg) of 30 wt% hydrogen peroxide in H_2O (5.0 equiv.) was added, and the resulting solution was stirred at rt for 30 min to yield TCA intermediates (36% isocitrate, 21% aconitate, and 37% succinate).

Conversion of oxaloacetate to α-ketoacid analogs (III, IV, V, VI, VII) followed by oxidation to TCA cycle intermediates:

1.321 g of oxaloacetic acid (10.0 mmol, 200 mM) was dissolved in 41.6 mL of a 0.50 M aq. pH 7 sodium phosphate buffer in a 100-mL round bottom. Then, 3310 μL (2221 mg) of 50 wt% glyoxylic acid in H_2O (3.0 equiv.) was added. The reaction pH was adjusted to 7.0 by adding 4.0 M aq. NaOH dropwise. The reaction was stirred at 50 °C for 21 h to yield α-ketoacid intermediates. Then, 408 μL (136 mg) of 30 wt% hydrogen peroxide in H_2O (5.0

equiv.) was added to a 4.0 mL reaction aliquot in a 20-mL vial, and the resulting solution was stirred at rt for 30 min.

Conversion of pyruvate (I) to alanine:

137 mg of glycine (1.83 mmol, 350 mM) was dissolved in 5.25 mL of deionized water in a 20-mL vial. Then, 241 mg of sodium pyruvate (1.2 equiv.) and 87 mg of aluminum potassium sulfate dodecahydrate (0.1 equiv.) was added. The reaction pH was adjusted to 5.0 by adding 4.0 M aq. NaOH dropwise and maintained by additions of 10% HCl. The reaction was stirred at 80 °C for 24 h to yield alanine in 19% yield.

Conversion of α-ketoglutarate (V) to glutamate:

137 mg of glycine (1.83 mmol, 365 mM) was dissolved in 5.0 mL of deionized water in a 20-mL vial. Then, 495 mg of disodium α-ketoglutarate (1.2 equiv.) and 87 mg of aluminum potassium sulfate dodecahydrate (0.1 equiv.) was added. The reaction pH was adjusted to 5.0 by adding 10% aq. HCl dropwise. The reaction was stirred at 80 °C for 4 h to yield glutamate in 44% yield.

Conversion of oxaloacetic acid (VIII) to citric acid:

132 mg of oxaloacetic acid (1.00 mmol, 200 mM) was dissolved in 4.5 mL of a 0.5 M aq. pH 7 sodium phosphate buffer in a 20-mL vial. Then, the reaction pH was adjusted to either 7.0 or 5.0 (Supplementary Fig. 3) by adding 4.0 M aq. NaOH dropwise. The reaction was stirred at 50 °C for 30 min, then, 511 μL (170 mg) of 30 wt% hydrogen peroxide in H_2O (5.0 equiv.) were added. The resulting reaction was stirred at 23 °C (rt) for 5 min to yield citrate in 1% yield or 5% yield (Supplementary Fig. 5).

Conversion of oxaloacetate (VIII) to maloyl formate (HKG/III, Supplementary Figs. 12, 13):

119 mg of oxaloacetic acid (0.90 mmol, 90 mM) was dissolved in 9.9 mL of a 1.0 M aq. pH 7 sodium phosphate buffer in a 20-ml sealed reaction vial under an air atmosphere. 119 μL (160 mg) of 50 wt% glyoxylic acid in H_2O (1.2 equiv.) was added by micropipette. The reaction pH was adjusted to 7.0 with 4.0 M aq. NaOH. The reaction was stirred at 25 °C for 3 h to give >98% HKG. HKG produced in this step was used directly for the next step (synthesis of di-DHKG).

Conversion of maloyl formate (HKG/III) to di-DHKG (Supplementary Figs. 12, 13):

119 μL (160 mg, 1.2 equiv.) of 50 wt% glyoxylic acid in H_2O was added to the above HKG reaction mixture (0.90 mmol), and the pH of the reaction mixture was adjusted to 7.0 with 4.0 M aq. NaOH. The reaction mixture was stirred at 50 °C for 24 h to produce di-DHKG.

Conversion of α-ketoglutarate (V) to $^{13}C2$ labeled α-ketoglutarate (Supplementary Fig. 3):

14.6 mg of α-ketoglutaric acid (0.1 mmol, 100 mM) was dissolved in 1 mL of 1 M aq. sodium bicarbonate buffer in a 5-mL sealed vial under an air atmosphere. Then, 9.4 mg of $^{13}C2$ labeled glyoxylic acid monohydrate (1.0 equiv.) was added. The reaction pH was adjusted to 8.6 with 4.0 M aq. NaOH, and the reaction was stirred at 50 °C for 14 days.

Control reaction of glyoxylic acid (Supplementary Fig. 14):

In a 5 mL sealed glass vial under an air atmosphere, $^{13}C2$ labeled glyoxylic acid monohydrate (9.4 mg, 100 mM) was dissolved in 1 mL of 1 M $NaHCO_3$ buffer and pH of the reaction mixture was adjusted to 8.0 with 2.0 M aq. NaOH. The reaction mixture was stirred at 50 °C for 10 days.

Data availability statement:

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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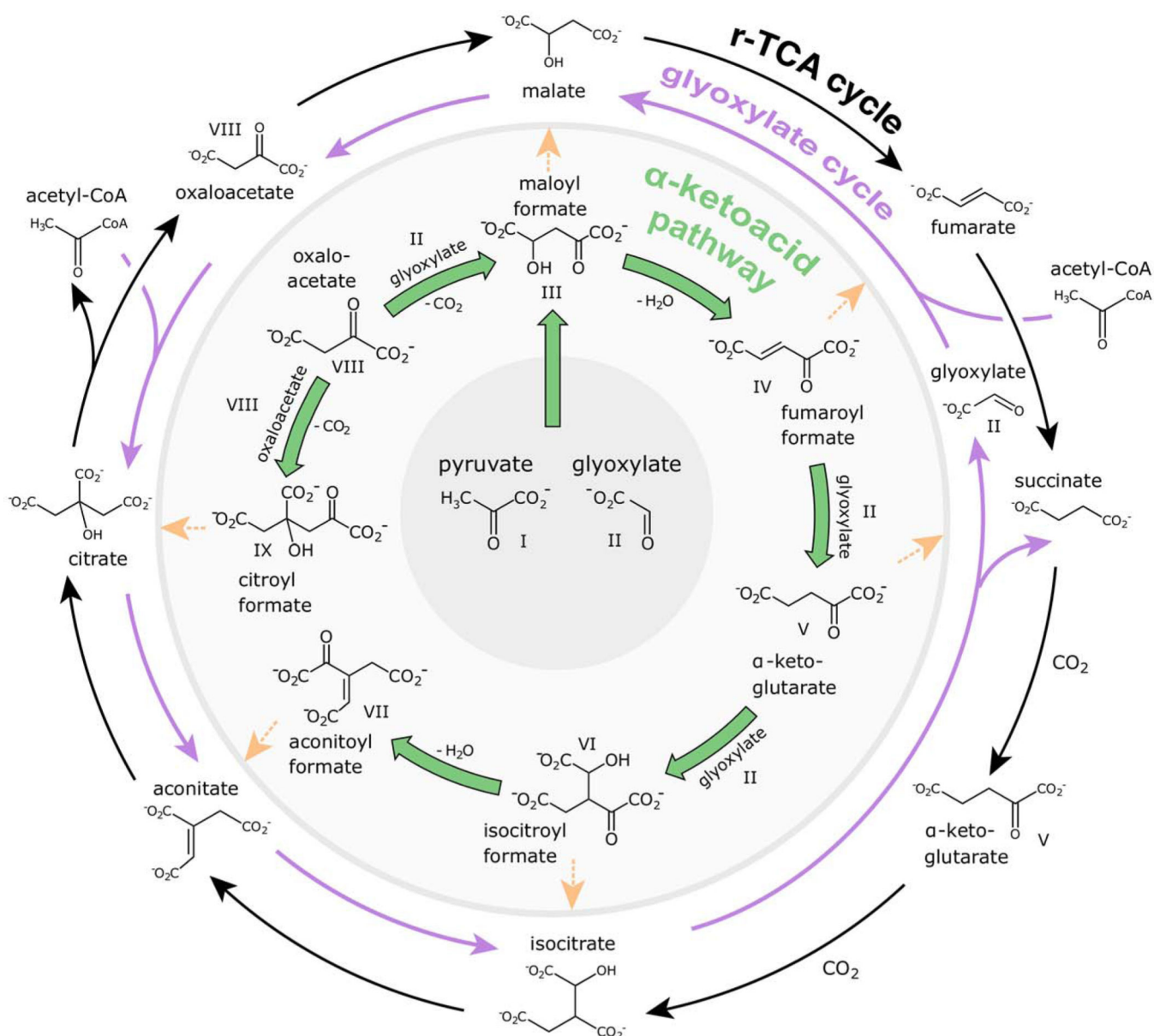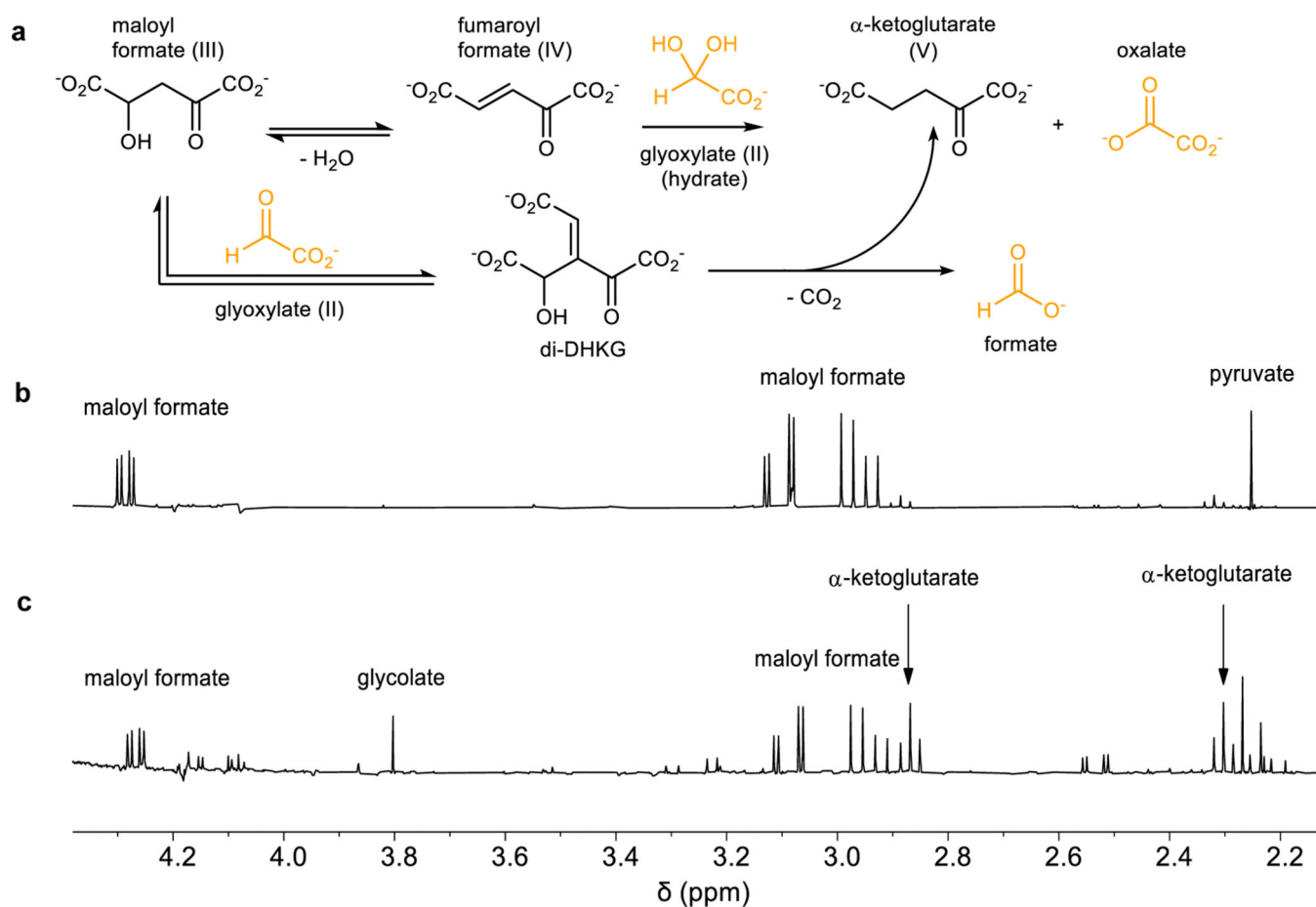
Figure 1.
The α-ketoacid pathway (inner green arrows) resembles transformations within the r-TCA (black) and glyoxylate (purple) cycles. Starting from pyruvate (I) or oxaloacetate (VIII), under mild aqueous conditions, the α-ketoacid pathway requires only the addition of glyoxylate (II), acting as both the carbon source and reductant, to progress through a series of α-ketoacid analogs of metabolic carboxylate intermediates. The glyoxylate cycle in modern biology similarly uses acetyl-CoA (in place of pyruvate) and glyoxylate as building blocks. Although likely a later evolutionary invention, intermediates of the α-ketoacid pathway can be transformed into their respective TCA cycle intermediates via oxidative decarboxylation in the presence of an appropriate oxidant (peach arrows).
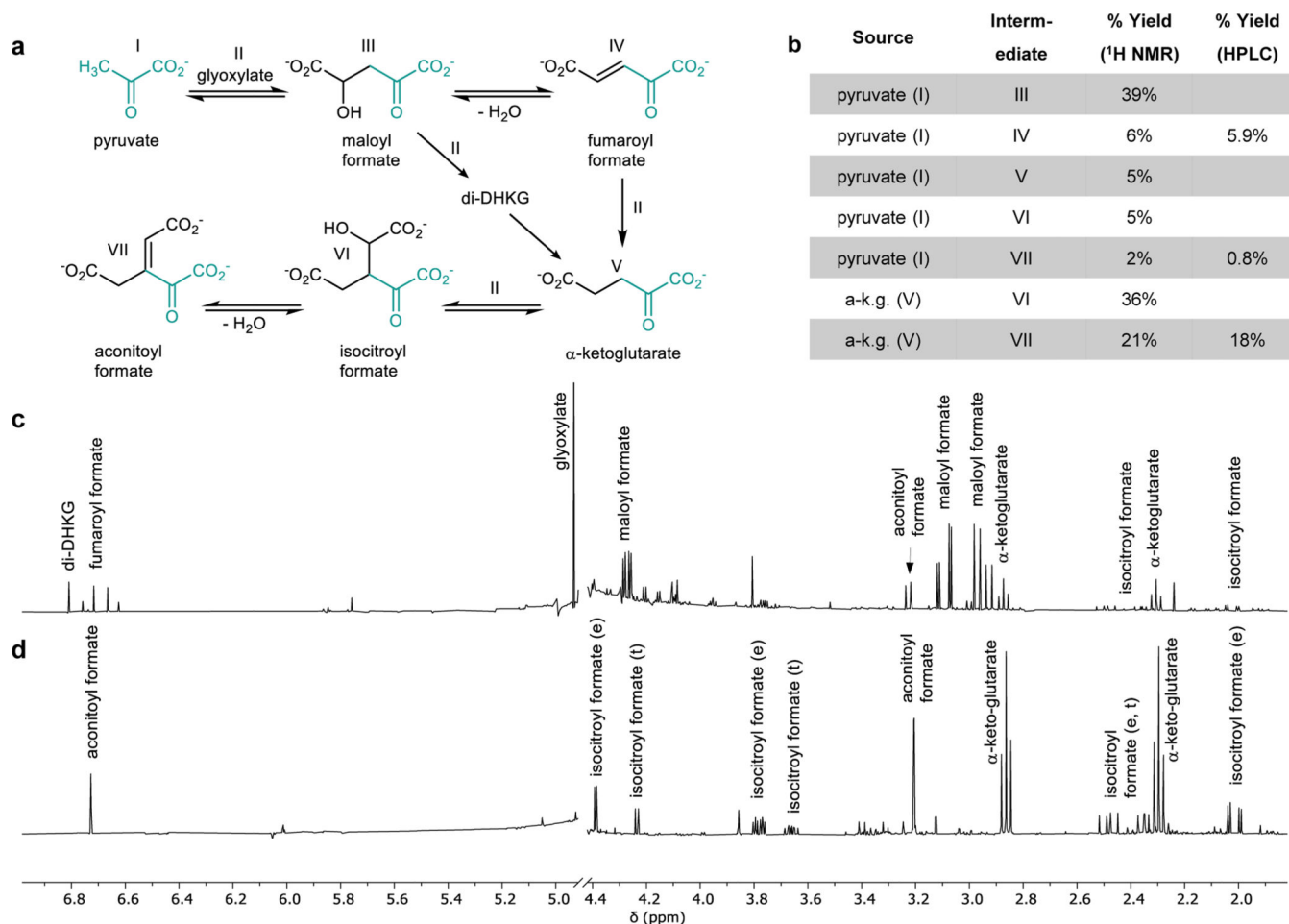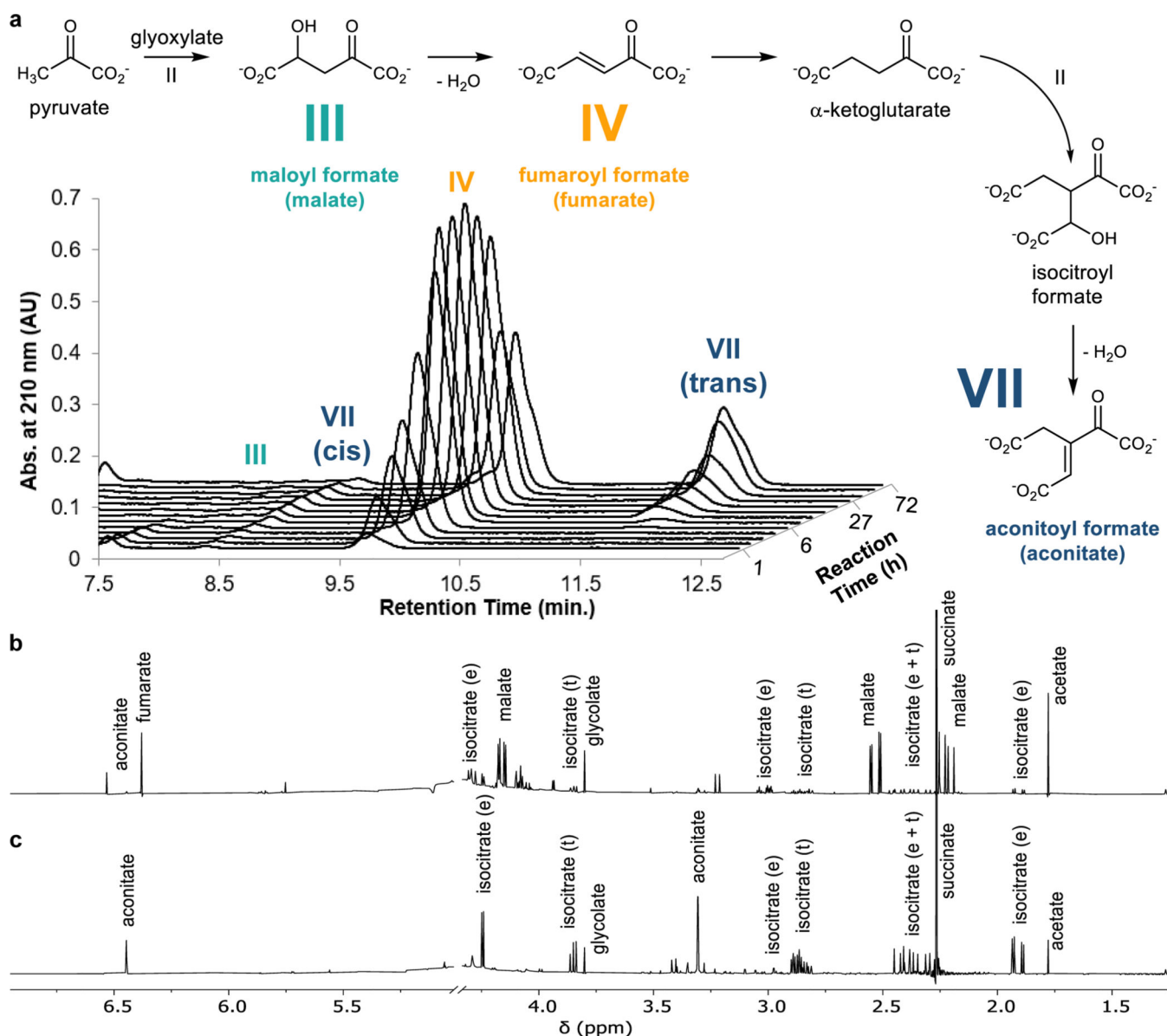
Figure 2.

The α-ketoacid analog pathway generated by the reaction of glyoxylate with pyruvate. (a) The reaction scheme highlights the pyruvate scaffold (in teal), a secondary pathway to α-ketoglutarate occurs through a di-DHKG intermediate (Supplementary Fig. 1), (b) Reaction yields from pyruvate and from α-ketoglutarate were determined by ^{1}H-NMR integration versus an internal *t*-BuOH standard and by HPLC-UV (at 210 nm) versus a standard curve. (C) ^{1}H NMR (in D_2O) of a 100 μl aliquot from a reaction of 200 mM pyruvate (I) and 3 equiv. of glyoxylate (II) in a 0.5 M phosphate buffer at pH 7 heated to 50 °C for 21 h, yields 39% maloyl formate (III), 6% fumaroyl formate (IV), 5% α -ketoglutarate (α -k.g., V), 5% isocitroyl formate (VI), 2% aconitoyl formate (VII) with 1% pyruvate remaining. (d) ^{1}H NMR (in D_2O) of a 100 μl aliquot from a reaction of 200 mM α-ketoglutarate (V) and 1 equiv. of glyoxylate (II) in a 0.5 M phosphate buffer at pH 7 heated to 50 °C for 21 h, yields 36% isocitroyl formate (VI) and 21% aconitoyl formate (VII) with 37% α-ketoglutarate remaining. Erythro (e) and threo (t) diastereomers of isocitroyl formate were identified by oxidation to isocitrate standards.

Figure 3.

The glyoxylate-dependent reduction of maloyl formate (III) to α-ketoglutarate (IV). (a) Two reaction pathways for the reduction of III to α-ketoglutarate (V) proceed through either IV (via a cross-Cannizzaro type hydride transfer to V), or di-DHKG (via decarboxylation and retro-Claisen fragmentation to V). Oxalate and formate side products formed from glyoxylate (highlighted in amber) provide evidence for the operation of both unique pathways. (b) ^{1}H NMR (in D_2O) of a 100 μl aliquot from a control reaction of 200 mM maloyl formate (III) in a 0.5 M pH 7 phosphate buffer heated to 50 °C for 21 h, (c) ^{1}H NMR (in D_2O) of a 100 μl aliquot from a reaction of 200 mM maloyl formate (III) and 1 equiv. glyoxylate (II) in a 0.5 M pH 7 phosphate buffer heated to 50 °C for 21 h. The maloyl formate (4-hydroxy-2-ketoglutarate, HKG) was obtained from a commercial source.
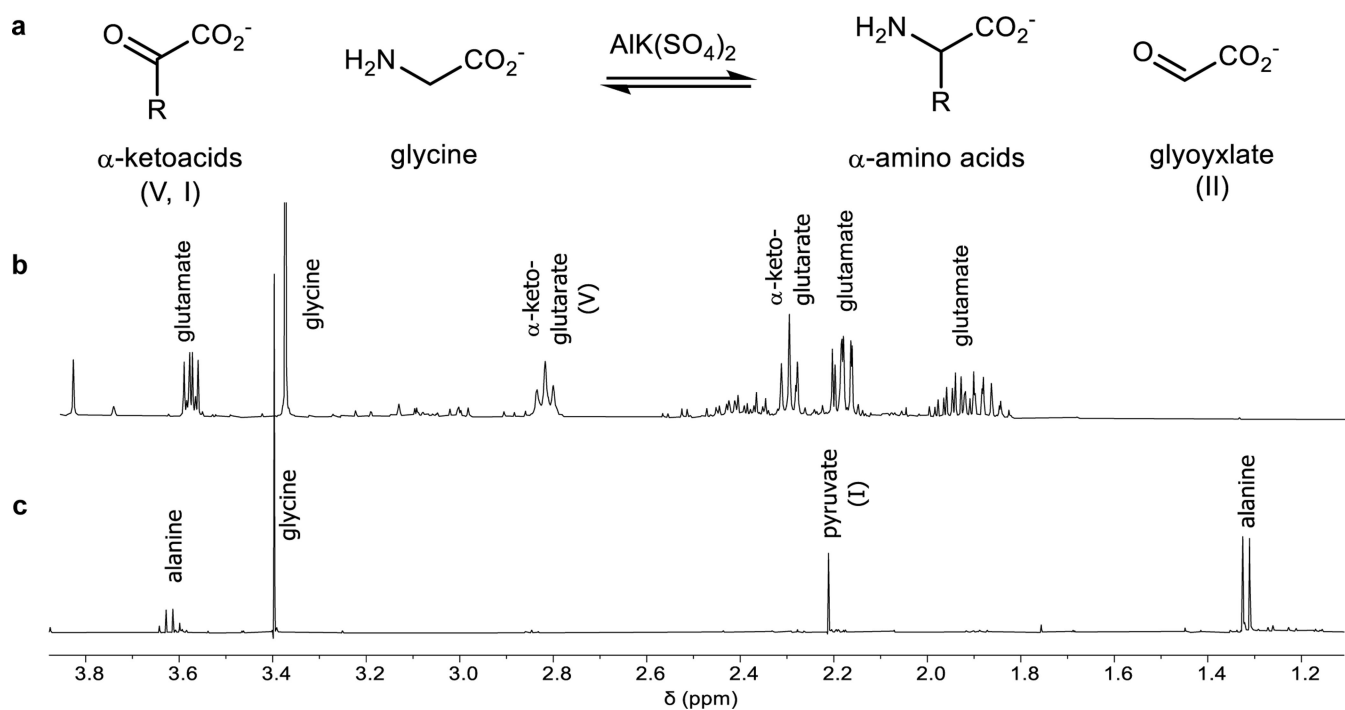
Figure 4.
Reaction progression of the α-ketoacid pathway with time. (a) The progression of the pyruvate and glyoxylate reaction sequence was visualized by an HPLC time course study after oxidation of reaction aliquots. 200 mM pyruvate with 3 equiv. of glyoxylate in a 0.5 M pH 7 phosphate buffer was heated to 50 °C for 1, 2, 3, 4, 6, 10, 14, 20, 27, 31, 35, 53, and 72 h, to yield 5.9% fumaroyl formate (IV) and 0.8% aconitoyl formate (VII) after 27 h, and 1.9 % fumarate and 2.3% aconitate after 72 h. Reaction aliquots were oxidized to the canonical r-TCA intermediates with the addition of 5 equiv. H_2O_2 stirred for 0.5 h at rt before analysis by HPLC/UV in comparison to seven-point standard calibration curves of the canonical standards. (b) ^1H NMR (in D_2O) of a 100 μl aliquot from a reaction of 200 mM pyruvate and 3 equiv. of glyoxylate in a 0.5 M pH 7 phosphate buffer heated to 50 °C for 21 h, followed by the addition of 5.0 equiv. of H_2O_2 and stirring for 0.5 h at rt. Erythro (e) and threo (t) diastereomers of isocitrate are labeled. (c) ^1H NMR (in D_2O) of a 100 μl

aliquot from a reaction of 200 mM α-ketoglutarate and 1 equiv. of glyoxylate in a 0.5 M pH 7 phosphate buffer heated to 50 °C for 21 h, followed by the addition of 5.0 equiv. of H_2O_2 and stirring for 0.5 h at rt.

Figure 5.
The transamination of α-ketoacids and glycine into α-amino acids and glyxoylate. (a) α-ketoglutarate (V) and pyruvate (I) produce glutamate and pyruvate respectively in the presence of glycine as the nitrogen source, (b) ^{1}H NMR (in D_2O) of a reaction of 350 mM glycine with 1.2 equiv. of α-ketoglutarate in a pH 5 aqueous solution with 0.1 equiv. of AlK(SO$_4$)$_2$ heated to 80 °C yields 44% glutamate after 4 h, (c) an equivalent reaction of glycine with pyruvate yields alanine in 19% yield after 24 h.