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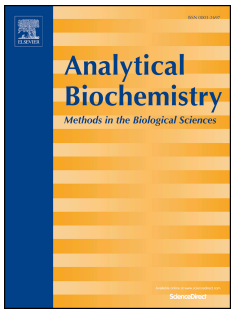

Synthesis of α-Ketoglutaramic Acid

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Maria Linder
Executive Editor
Analytical Biochemistry

Dear Dr. Linder,

These are the Author Contributions – CrediT for the manuscript titled: Synthesis of α-Ketoglutaramic Acid

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Laken Kruger: Data curation, Writing- Reviewing and Editing

Tyler Deatherage: Data curation, Investigation, Formal Analysis, Writing- Reviewing and Editing

Travis T. Denton, Ph.D.: Funding Acquisition, Project administration, Supervision, Visualization, Conceptualization, Methodology, Writing- Original draft preparation, Writing- Reviewing and Editing.

Sincerely,

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Synthesis of α-Ketoglutaramic Acid

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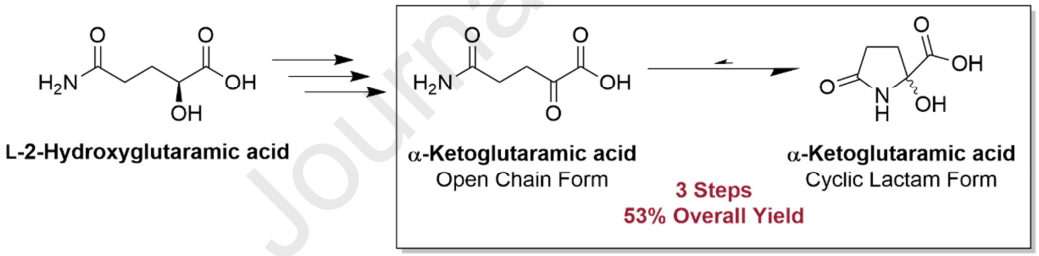
Abstract

α-Ketoglutaramic acid (KGM, α-ketoglutaramate), also known as 2-oxoglutaramic acid (OGM, 2-oxoglutaramate), is a substrate of ω-amidase, also known as Nitrilase 2 (NIT2), and is essential for studying the canonical role of ω-amidase, as well as its role in multiple diseases. Until now, KGM used for biological studies has been prepared most often by the enzymatic oxidation of L-glutamine using snake venom L-amino acid oxidase, which provides KGM as an aqueous solution, containing by-products including 5-oxoproline and α-ketoglutarate. The enzymatic method for KGM preparation, therefore, cannot provide pure product or an accurate percent yield evaluation. Here, we report a synthetic method for the preparation of this important substrate, KGM, in 3 steps, from L-2-hydroxyglutaramic acid, in pure form, in 53% overall yield.

Graphical Abstract



Keywords

ketoglutaramic, ketoglutaramate, oxoglutaramic, oxoglutaramate, glutaminase, glutamine, glutamate, ω-amidase

1. Introduction

Glutamine, the most abundant amino acid in the human body, is a conditionally essential amino acid, which plays multiple roles in canonical biochemistry such as serving as a building block for proteins and as the main molecular source of brain derived ammonia. Until recently, glutamine had been underestimated in its role in conditions such as oxidative stress and cancer [1-21]. Many cancers reprogram their metabolism such that the ATP used to drive biochemical reactions is derived from glycolysis instead of the TCA cycle [22]. This type of cellular

reprogramming has been classically known as the Warburg effect [23]. Under these conditions, cancer cells reprogram their metabolism to rely more heavily on the TCA cycle intermediate α-ketoglutarate (KG), derived from glutamine, as the substrate for the production of succinyl-CoA, via the oxidative decarboxylation catalyzed by the α-ketoglutarate dehydrogenase complex (KGDHC) [24, 25]. Conversely, the cytosolic isocitrate dehydrogenase 1 (IDH1) and mitochondrial IDH2 catalyze the reductive carboxylation of KG by NADPH and CO_2 to produce isocitrate, which is used for the generation of nucleic acids, lipids and other building blocks necessary for the rapid proliferation of cancer cells. The KG used in these processes is produced from glutamine via two independent pathways termed the glutaminase I pathway (**Figure 1**, lower, blue pathway) and the glutaminase II pathway (**Figure 1**, upper, pink pathway). In the glutaminase I pathway, glutamine is converted to glutamate by enzymatic hydrolysis via glutaminase (GLS). The intermediate glutamate is either oxidatively deaminated to α-ketoglutarate via glutamate dehydrogenase or transaminated via an L-glutamate utilizing aminotransferase (transaminase) [26-28]. In the glutaminase II pathway, glutamine is transaminated by glutamine transaminase K, or glutamine transaminase L, to yield α-ketoglutaramate (KGM), which is ultimately hydrolyzed to KG by ω-amidase (NIT2, Figure 1) [23, 29-33].

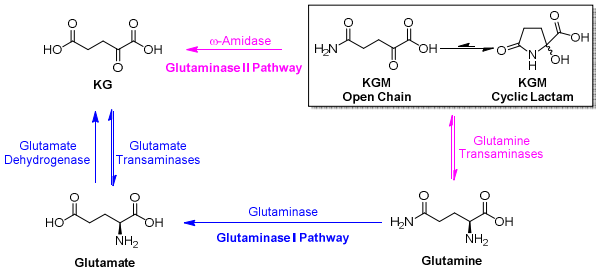


Fig. 1. KGMs role in the glutaminase pathways.

The majority of studies on glutamine anaplerosis are focused on the glutaminase I pathway, specifically, the selective inhibition of glutaminase [7, 13, 14, 34]. There are two main glutaminase isozymes, the kidney isozyme (KGA or GLS) and the liver isozyme (LGA or GLS2). Additionally, full length GLS (KGA) has a lower molecular weight, splice variant termed kidney glutaminase isoform C (GAC). GAC is the isoform of GLS found to be especially responsible for glutamine addiction in cancer [35]. Indeed, the potent, allosteric inhibitor of GAC, CB-839, has been utilized as a monotherapy for cancer treatment, but failed to be effective without support of an additional chemotherapeutic [34, 36, 37]. For example, the combination of CB-839 with known chemotherapeutic agents such as talazoparib for patients with advanced / metastatic solid tumors; nivolumab for patients with clear cell renal cell carcinoma, melanoma, and non-small cell lung cancer; palbociclib in patients with solid tumors; osimertinib in treating patients with EGFR-mutated stage IV non-small cell lung cancer and multiple others have progressed to Phase I/II clinical trials [38] [39] [40] [41-44]. The failure of CB-839 as a chemotherapeutic alone suggests that, upon inhibition of GLS, an alternative route for glutamine utilization may be exploited, such as the glutaminase II pathway [45]. Therefore, potent and selective inhibitors of the enzymes involved in the glutaminase II pathway may have the potential as new chemotherapeutic agents. The key intermediate molecule in the glutaminase II pathway, KGM, and its role in cancer cell biology has been understudied due, in part, to the lack of a straightforward synthetic method for the preparation of this key compound [46-49]. KGM has been detected in normal human cerebrospinal fluid (CSF), normal human urine and rat tissues (brain, liver and kidney) in the μM to >10 μM range [20, 48, 50, 51].

Besides its involvement in cancer biology, abnormal KGM levels *in vivo* are found in several hyperammonemic diseases [20, 47, 49, 52]. For example, the KGM levels in the CSF of patients, who have liver disease, correlate to the degree of hepatic encephalopathy (HE) [51, 52]. Deficiency of citrin, the hepatic isoform of the mitochondrial aspartate–glutamate carrier (*SLC25A13*) is associated with increased urine levels of KGM, reflective of secondary hyperammonemia [53-55]. Part of the excess glutamine is transaminated to KGM, which correlates with the degree of HE [47, 51]. Additionally, defects of the urea cycle cause an increase of KGM in the urine of patients with hyperammonemia [20].

A synthetic method for the preparation of KGM, in good yield and high purity is needed. To date, the most readily utilized preparation for KGM is the oxidation of L-glutamine by snake venom derived L-amino acid oxidase, which is expensive and affords KGM as an impure aqueous solution, contaminated with 5-oxoproline and α-ketoglutarate, which are difficult to separate from KGM using traditional, preparative scale techniques [23].

The use of pure KGM is vital for the metabolomic studies of not only the Glutaminase II pathway, but as a biomarker for the correlation of the degree of HE, hyperammonemic diseases due to the inborn error of citrin, defects of the urea cycle and as a potential biomarker for the identification of glutamine-addicted cancers. Determination of the specific role of ω-amidase in cancers is important to cancer biology and the development of small molecules as inhibitors of ω-amidase may provide an additional pathway in the development of chemotherapeutic agents for the treatment of multiple forms of cancer [31, 45]. Additionally, in order to understand the biochemistry of ω-amidase from biological sources, the kinetics and thermodynamics of the reaction utilizing the natural substrate, KGM, in place of the typically used substrate succinamic acid, or impure KGM, are necessary.

2. Materials and Methods

2.1 General Procedures

Unless otherwise noted, all materials for the synthetic chemistry were obtained from VWR and used without further purification. Microwave reactions were carried out in a Biotage Initiator^{+} system. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer using $CDCl_3$ or D_2O as the solvent. Chemical shifts (δ) for ^{1}H NMR are reported in ppm relative to the residual solvent signal (7.26 ppm, $CDCl_3$, 4.63 ppm, D_2O), coupling constants (*J*) are reported in hertz and peak multiplicities are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or br s (broad singlet). Thin layer chromatography (TLC) was performed on glass backed, 0.20 mm silica gel MF254 plates (Agela Technologies, USA), flash column chromatography was performed using KP-SIL silica gel (Biotage, USA) and automated flash column chromatography was performed on Biotage prepacked columns using the Biotage Isolera One (Biotage AB, Uppsala, Sweden). ^{1}H NMR, ^{13}C NMR and mass spectra were consistent with the assigned structures.

2.2. Chemical Synthesis

3-(Trifluoromethyl)benzyl (S)-5-amino-2-hydroxy-5-oxopentanoate (**2**). To a solution of **1** (1.0056 g, 6.8352 mmol) in DMF (4 mL), at 0°C (external ice bath) in a 2.0-5.0 mL microwave reaction vial, containing a magnetic stir bar, was added *N*,*N*-diisopropylethylamine (1.2426 mL, 7.5188 mmol) via micropipette, the mixture was vigorously mixed by pipetting up and down followed by magnetic stirring with the aid of an external stir plate. To the stirring solution was added 3-(trifluoromethyl) benzyl bromide (1.0544 mL, 6.8036 mmol), in one portion, by micropipette. The microwave reaction vial was purged with argon, capped and the solution was irradiated for 10 minutes at 60 °C. After cooling to room temperature, the sealed cap was removed, the stir bar was removed and contents of the vial were transferred to a 60 mL separatory funnel with the aid of ethyl acetate (50 mL). The organic layer was washed once with 0.1 M hydrochloric acid (50 mL) and twice with saturated sodium chloride (50 mL). The organic layer was transferred to a 250 mL Erlenmeyer flask and dried over excess sodium sulfate. The solids were removed via gravity filtration through fluted filter paper, the bulk solvent was removed by rotary evaporation and the trace solvent and residual benzyl bromide was removed by high vacuum to afford **2** as a clear, viscous oil (1.6129 g, 77.3% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.40 - 7.70 (m, 4H), 5.61 (br. s., 2H), 5.20 - 5.29 (m, 2H), 4.23 - 4.40 (m, 1H), 3.61 (d, *J* = 5.4 Hz, 1H), 2.31 - 2.47 (m, 2H), 2.22 (m, 1H), 1.93 - 2.03 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ = 178.3, 170.9, 136.2, 131.6, 131.1 (q, $J_{C\text{-}F}$ = 37.8 Hz), 129.3, 125.3 (m), 124.9 (m), 124.9 (q, $J_{C\text{-}F}$ = 277.2 Hz,), 69.8, 66.3, 31.0, 29.3; HRMS (ESI) *m*/*z* calcd for $C_{13}H_{15}F_3NO_4$ $[M + H]^+$ 306.0953, found 306.0961.

3-(Trifluoromethyl)benzyl 2-hydroxy-5-oxopyrrolidine-2-carboxylate (**3**). To a solution of **2** (4.4356 g, 14.531 mmol), in dichloromethane (35 mL), was added solid Dess-Martin periodinane (6.7796 g, 15.9841 mmol), in portions, over one minute. To the reaction mixture was added 1 drop of deionized water and the resultant mixture was allowed to stir at room temperature for 4 hours. The stir bar was removed and the contents of the round bottom flask were transferred to a 500 mL separatory funnel with the aid of ethyl acetate (100 mL). This solution was washed once with water (100 mL) and twice with a 1:1, saturated sodium bicarbonate / 5% sodium thiosulfate-pentahydrate mixture (100 mL). The organics were transferred to a 250 mL Erlenmeyer flask and dried over excess sodium sulfate. The solids were removed via gravity filtration through fluted filter paper, the bulk solvent was removed by rotary evaporation and the crude mixture was purified by flash chromatography (gradient elution: 2% - 20% methanol in dichloromethane) to afford **3**, (3.2333 g, 73.4% yield) as a light yellow, viscous oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.43 - 7.75 (m, 4H), 6.25 (br. s., 1H), 5.20 - 5.42 (m, 2H), 3.86 - 4.07 (br. s., 1H), 2.51 - 2.68 (m, 2H), 2.39 - 2.50 (m, 1H), 2.20 - 2.32 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ = 178.3, 170.87, 135.6, 131.5, 131.0 (q, $J_{C\text{-}F}$ = 32.8 Hz), 129.3, 125.5 (q, $J_{C\text{-}F}$ = 3.8 Hz), 124.9 (q, $J_{C\text{-}F}$ = 3.8 Hz), 123.8 (q, $J_{C\text{-}F}$ = 273.4 Hz), 86.9, 67.2, 32.6, 29.3; HRMS (ESI) *m*/*z* calcd for $C_{13}H_{13}F_3NO_4$ $[M + H]^+$ 304.0797, found 304.0803.

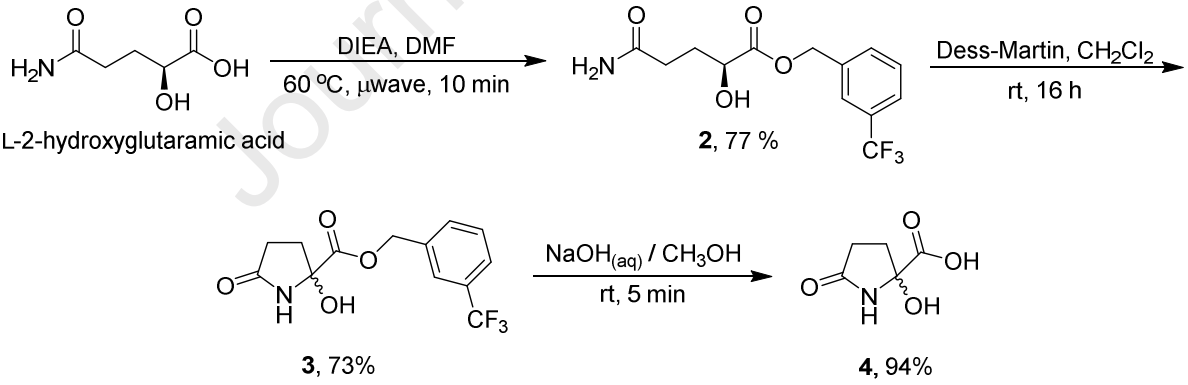
2-Hydroxy-5-oxopyrrolidine-2-carboxylic acid (**4**). To a solution of **3** (209.5 mg, 0.6902 mmol) in methanol (10 mL) was added $NaOH_{(aq)}$ (1 M, 1.381 mL, 1.381 mmol) in one portion. The resultant mixture was allowed to stir until no starting material was evident by TLC (5 minutes). The volatiles were removed *in vacuo* and the residue was dissolved in water (5 mL), transferred

to a 50 mL centrifuge tube containing 5 equivalents (10 mL) of Dowex-50WX8 ion exchange resin (H^+ form) and mixed by inversion for 20 minutes. The resin was removed via gravity filtration using a fritted chromatography column, the resin was washed with ca. 3 column volumes of water and the combined filtrates were collected in a separatory funnel, which was washed with ethyl acetate (3 x 20 mL), transferred to a 100 mL round bottom flask and volatiles were removed *in vacuo* to afford **4** (93.7 mg, 93.62% yield) as opaque brown crystals: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.37 - 2.53 (m, 2H), 2.32 (m, 1H), 1.96 - 2.09 (m, 1H); ^{13}C NMR (75 MHz, D_2O) δ 181.6, 174.9, 88.1, 33.6, 29.6; HRMS (ESI) *m/z* calcd for $C_5H_8NO_4$ $[M + H]^+$ 146.0453, found 146.0453, $C_5H_6NO_3$ $[M - OH]^+$ 128.0342, found 128.0346.

3. Results and Discussion

The current synthetic strategy for the preparation of KGM is outlined in Scheme 1. To allow for organic solvent solubility, and afford a protecting group that will also serve as a pro-drug moiety, L-2-hydroxyglutaramic acid (**1**, L-2-HGM, hereafter referred to as HGM), prepared by our previously optimized nitrous acid oxidation of L-glutamine, was esterified to compound **2**, in 77% yield, by treatment of the carboxylate with 3-(trifluoromethyl)benzyl bromide in DMF under microwave irradiation [23]. Next, the alcohol was oxidized by Dess-Martin periodinane to yield the ketone, which spontaneously cyclizes to afford compound **3**, in 73% yield. Finally, the deprotection of the ester, by treatment with aqueous sodium hydroxide in methanol, followed by protonation with H^+ form ion exchange resin, afforded KGM in 94% yield.

Scheme 1. Synthetic Strategy for the Preparation of KGM

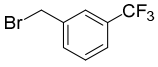
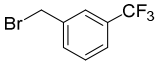
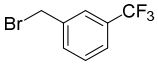
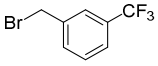
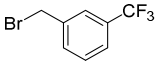
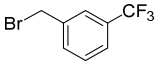
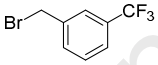
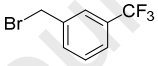
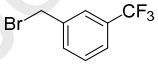
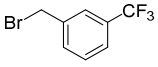
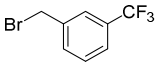
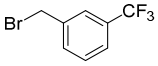
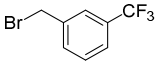


The most straightforward method to obtain KGM would be the aqueous oxidation of HGM. However, the strong oxidation conditions tend to favor decarboxylation of the α-keto acid product. Oxidations with Jones reagent, $KMnO_4$ or polymer bound chromium trioxide either led to the decomposition of the products or to product mixtures that were inseparable from solubilized chromium species, as evidenced by the line broadening in the NMR spectra. The use of Fremy's salt ($K_2NO(SO_3)_2$) leads to noticeable amounts of KGM as deduced by NMR

analysis, but the amount of salt required (at least 9 molar equivalents), time required for the reaction to reach completion (weeks to months) and the separation/isolation of the KGM from the salt mixture makes this method impractical [56]. In a patented procedure using Fremy's salt, the process is not detailed, difficult to reproduce, the product is not characterized and the yield is unsubstantiated [57]. Due to the difficulty of over-oxidation and the separation of the KGM product from the salt by-products, we turned to a protection/deprotection strategy. Although minimization of the use of protecting groups in organic synthesis is paramount, in medicinal chemistry, a well-designed synthesis, incorporating the "right" protecting groups, can provide a useful strategy for the synthesis of the desired end product, while providing useful prodrug forms along the way. We decided to esterify HGM to protect the carboxylic acid from decarboxylation during oxidation of the 2-hydroxyl group to the α-keto group, while also providing solubility in organic solvents to take advantage of the more subtle, dichloromethane soluble, oxidizing agent, Dess-Martin periodinane, for the preparation of the KGM ester (**3**, Scheme 1).

As a result of the difference in polarity between HGM and the alkylating agents, iodoethane and 3-(trifluoromethyl)benzyl bromide, the esterification of HGM was determined to be impractical in a number of typical alkylating systems (Table 1). Treatment of the cesium salt of HGM with ethyl iodide provided no products, as determined by TLC analysis, after 22 hours in refluxing solvent (entry I). Realizing that more lipophilic esters would be more valuable as prodrugs, attention was focused on the use of (3-(trifluoromethyl)benzyl bromide (entry II) for 2 hours in refluxing acetone, after which no new spots were visualized by TLC. Realizing that the dissimilarities in the physical properties of the starting materials was prohibiting the reaction, the solvent was changed to DMF and the cesium salt of HGM was used, to increase both the solubility of the alkylating agent and the electrophile. Using these conditions, the product was afforded, in 16% yield, after 18 hours at room temperature (entry III). Utilizing the sodium salt form of HGM, the reaction proceeded equally as well under the same conditions (entry IV). Heating the alkylation reaction to 60 ^{o}C showed no increase in the yield when utilizing the sodium salt form of HGM (entry V) and a decreased yield was observed for the cesium salt after 10 min of microwave irradiation at 60 ^{o}C in the presence of (3-(trifluoromethyl)benzyl bromide (entry VI). In an effort to avoid a separate isolation step, the deprotonation of the acid form of HGM, *in situ*, was performed in DMF and DMSO utilizing the prototypical bases cesium carbonate and or potassium carbonate (entries VII-X). However, these reactions were less than optimal. The combination of the poor reactivity of the cesium or sodium salt of HGM and the limited solubility of the cesium or potassium carbonate in the solvent, rendered this system impractical for the transformation. In an attempt to avoid solubility issues of the base used for deprotonation or the conjugate base/acid pair, in the solvent, we employed diisopropylethylamine, in DMF, under microwave irradiation, at 60 ^{o}C, for ten minutes, which resulted in a 77% yield of the desired product, after chromatography (entry XI). In an attempt to increase the yield; temperatures, reaction times and solvent were adjusted (entries XII-XIV). It was determined that the conditions of entry XI were optimal.

Table 1. Optimization of the esterification of HGM

#	HGM Form	R-LG (eq)	Solvent	Reaction Condition	Base	Time	% Yield
I	Cs^+	 (1.1)	1:1 water/ dioxane	RT	N/A	22 hrs	0
II	Na^+	 (1.1)	Acetone	Reflux	N/A	2 hrs	0
III	Cs^+	 (1.5)	DMF	RT	N/A	18 hrs	16.3
IV	Na^+	 (1.1)	DMF	RT	N/A	18 hrs	36.9
V	Na^+	 1.5	DMF	60 °C hot plate	N/A	4 hrs	31.3
VI	Cs^+	 (1.1)	DMF	60 °C microwave	N/A	10 min	15.6
VII	H^+	 (1.1)	DMF	RT	Cs_2CO_3	18 hrs	0
VIII	H^+	 (1.1)	DMSO	60 °C hot plate	Cs_2CO_3	18 hrs	0
IX	H^+	 (1.1)	DMF	60 °C microwave	Cs_2CO_3	10 min	0
X	H^+	 (1.1)	DMSO	60 °C hot plate	K_2CO_3	18 hrs	0
XI	H^+	 (1.1)	DMF	60 °C microwave	DIEA	10 min	77.3
XII	H^+	 (1.1)	DMF	RT	DIEA	18 hrs	59.3
XIII	H^+	 (1.1)	DMF	100 °C microwave	DIEA	10 min	62.4
XIV	H^+	 (1.1)	DMSO	100 °C microwave	DIEA	10 min	58.3

The solubility of the ester in dichloromethane made the oxidation of the secondary alcohol straightforward using Dess-Martin periodinane to afford the KGM ester **3** in 73.4% yield after chromatography. The KGM ester was saponified using $NaOH_{(aq)}$ in methanol, followed by protonation with ion exchange resin and removal of the by-products by liquid-liquid extraction, to afford pure KGM, in 94% yield, as an opaque brown solid.

4. Conclusion

In conclusion, this enzyme-free method for the production of KGM on a preparative scale will provide a necessary, highly pure form of the molecule needed to study the role of the Glutaminase II pathway in human diseases. Obtaining pure KGM will also allow for the utilization of the biologically relevant enzymatic transformation when employing a medicinal chemistry campaign to identify potent ω-amidase inhibitors and activators. During the synthesis, the esterification step not only protects against decarboxylation during oxidation, but also provides a potential prodrug of KGM that will increase the cell permeability and efficiency of the molecule in *in vitro* assays. In the procedure described, KGM is produced in high yield and exceptional purity as compared to all alternative preparations of KGM.

5. Notes

The authors declare no conflict of financial interests.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. D. Shen was the first to synthesize KGM. D. Shen, L. Kruger and T. Deatherage each contributed to the optimization of the reactions. T. T. Denton designed the synthesis, wrote and edited the manuscript and coordinated the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

The supporting information containing ^{1}H and ^{13}C NMR spectra for the new compounds (PDF).

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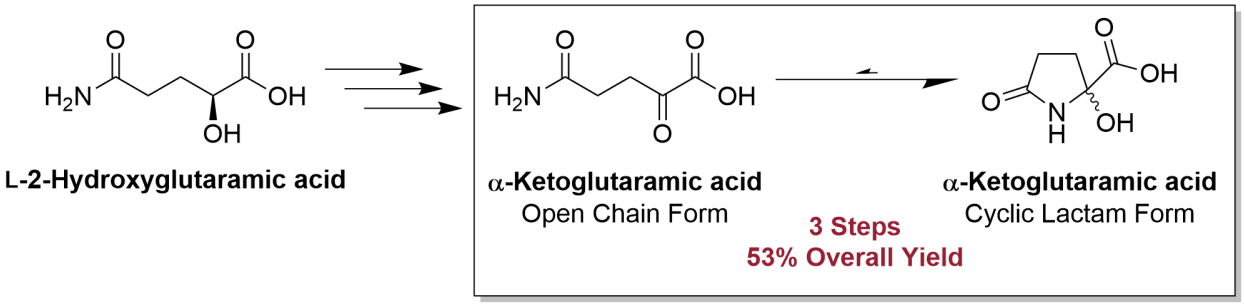

Synthesis of α-Ketoglutaramic Acid

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Highlights

- α-Ketoglutaramate was synthesized in 58% overall yield
- Prodrug forms of α-ketoglutaramate can be built into the synthesis
- α-Ketoglutaramate is a natural substrate of omega-amidase
- Pure α-ketoglutaramate allows for the study of the Glutaminase II pathway

List of Abbreviations:

ATP Adenosine Triphosphate

CO_2 Carbon Dioxide

CB-839

CSF Cerebrospinal Fluid

DIEA Diisopropyl Ethylamine

DMF Dimethylformamide

DMSO Dimethylsulfoxide

EGFR Epidermal Growth Factor Receptor

GLS Glutaminase

HE Hepatic Encephalopathy

IDH1 Isocitrate Dehydrogenase 1

KG α-Ketoglutarate

KGDHC α-Ketoglutarate Dehydrogenase Complex

KGM α-Ketoglutaramic acid

μwave Microwave

NaOH Sodium Hydroxide

NADPH Nicotinamide adenine dinucleotide phosphate

NMR Nuclear Magnetic Resonance

NIT2 Nitrilase 2

OGM 2-oxoglutaramic acid

rt Room Temperature

TCA Tricarboxylic Acid

TLC Thin Layer Chromatography