# Glutamic Acid-1-semialdehyde, a Hypothetical Intermediate in the Biosynthesis of 5-Aminolevulinic Acid

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Whether glutamate-1-semialdehyde (G-1-SA) or 4,5 dioxovalerate (DOVA) is the intermediate between glutamate and ALA in the C-5 tetrapyrrol pathway is still a matter of controversy. Since no data characterizing G-1-SA are available it is not possible either to identify G-1-SA as intermediate or a precursor of ALA. Therefore, attempts were made to establish a chemical synthesis of this compound. Two strategies were developed: starting from protected glutamate *via* an open chain mechanism or from the cyclic compound pyroglutamic acid or derivatives. None of the attempts were successful. Both approaches yielded undefined polymeric products when it was tried to liberate G-1-SA from the last intermediate of the synthesis sequence.

## Introduction

The first well characterized intermediate of natural porphyrins such as heme or chlorophylls is 5-aminolevulinic acid (ALA). The formation of ALA *via* the classical Shemin-pathway is clearly understood [1], whereas the intermediate between glutamate and ALA in the C-5 pathway is still a matter of controversy. As most probable intermediates 4,5-dioxovaleric acid (DOVA) and glutamate-1-semialdehyde (G-1-SA) are favoured (Fig. 1). DOVA has been chemically characterized [2] isolated from plant material [2–4] and demonstrated to be a substrate of an enzymic system for ALA-synthesis in algae and higher plants [3, 5–7]. Nevertheless there is still the discussion whether DOVA might be a degradation product of ALA [8]. G-1-SA is believed to be the substrate for a RNA containing enzyme system in barley and *Chlamydomonas reinhardtii* [9–11], although it has neither ever been isolated from any plant material nor has it been synthesized beyond doubt. It has been claimed several times that it was synthesized and used as precursor for ALA biosynthesis [12, 13], but only the product before the final step of its synthesis has been characterized in this

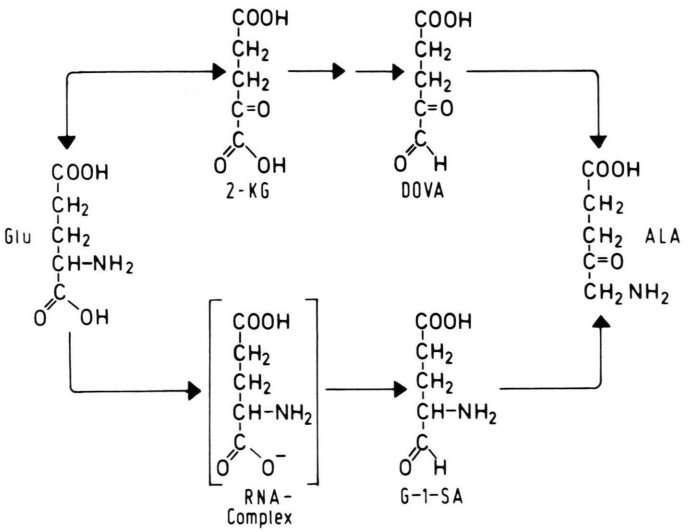


Fig. 1. The two *in vivo* pathways to 5-aminolevulinic acid (ALA) both starting from glutamate (Glu), 4,5-dioxovaleric acid (DOVA) and glutamate-1-semialdehyde (G-1-SA) are the controversely discussed intermediates. The chemical synthesis of DOVA is known, efforts to synthesize G-1-SA are described in this paper.

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paper [13]. Attempts by other authors to synthetize G-1-SA have also failed [14]. Thus the existence of free G-1-SA as a precursor of ALA remains purely hypothetical until its synthesis can be proven beyond doubt and the substance can be chemically and spectroscopically characterized for comparison.

In this paper we report about the attempts to synthesize G-1-SA *via* various synthesis sequences.

## Results and Discussion

Whereas DOVA has been synthesized and characterized [2] a synthesis of free glutamate-1-semialdehyde (G-1-SA) was reported by Kannangara and Gough [12, 13] in which G-1-SA was neither isolated nor characterized clearly. Only an aqueous solution containing G-1-SA was described. This solution was obtained from the reaction sequence which is outlined in Fig. 2. The starting material of this sequence which is easily prepared in two steps from glutamic acid [15, 16] was reacted with phosphorous pentachloride. The reaction product although not being characterized was stated to be acid chloride (**2**) and was submitted to a Rosenmund reduction [17].

During the latter reaction reduction of the acid-chloride function of **2** to an aldehyde and removing of the benzylic protective groups should be performed in one step.

To characterize the intermediates and reaction products we decided to reproduce the way of Kannangara and Gough. Therefore **1** was prepared in the usual manner [15, 16] and treated with phosphorous pentachloride as reported by the authors [12]. The reaction yielded a compound containing no chlorine and also lacking the signal of one of the benzyl groups in the $^1$H NMR spectrum. Both, combustion analysis and $^1$H NMR spectrum, suggest structure **5** (Fig. 3), which was confirmed by data from literature [15, 18].

We also submitted mixed anhydride **5** to Rosenmund reduction according to the procedure described by Kannangara and Gough [12]. We analyzed the aqueous reaction solution by thin layer and paper-chromatography [12], but only found glutamic

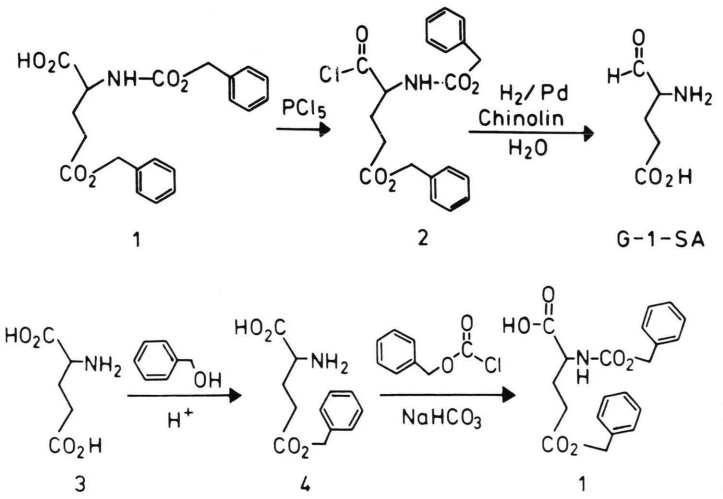


Fig. 2. The hypothetical reaction sequence to G-1-SA, starting from γ-benzyl-N-carbobenzyloxy-L-glutamate (**1**) *via* γ-benzyl-N-carbobenzyloxy-L-glutamic acid chloride (**2**), as described by Kannangara and Gough [9, 12].

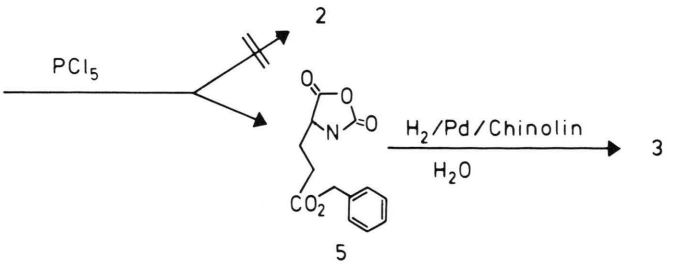


Fig. 3. Variation of the open chain strategy *via* the acid chloride, (**2**) however, is not formed. Only glutamic acid can be recovered.

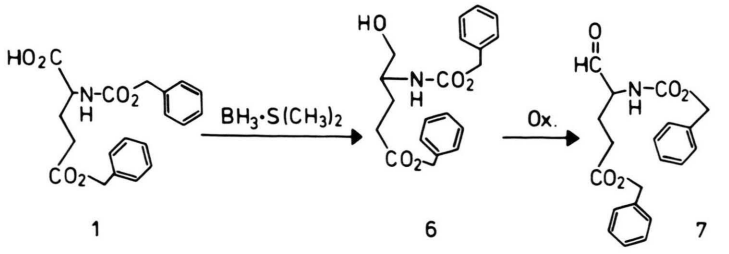


Fig. 4. Reduction of γ-benzyl-N-carbobenzyloxy-L-glutamate (**1**) by borane-dimethylsulfide complex to the corresponding alcohol (**6**) and subsequent oxidation to the aldehyde (**7**) by pyridinium dichromate, which failed.

acid and slight amounts hydrolysate **4**. Additional examination of our aqueous reaction solution by a separation procedure on a Dowex 50 WX 8-column according to Kannangara and Gough [12] gave no detectable aldehyde reaction in any of the fractions eluted from the column.

A hydrogenated sample being gratefully placed to our disposal by Dr. Kannangara, Carlsberg Institute, Kopenhagen, was analyzed in parallel to our examinations, but showed the same results.

The findings stated above are severely endoubting the existence of G-1-SA in the preparation of Kannagara and Gough. It has therefore been important to undertake efforts in preparing G-1-SA and to investigate its stability to get a standard preparation for enzymatic tests for further investigation of *in vitro* transformation of G-1-SA.

A convenient starting material to prepare G-1-SA is glutamic acid. The synthesis requires a selective functional group for interconversions of the α-carbonic acid group to an aldehyde function. To avoid condensation reactions it is necessary to protect both, γ-carboxylic and amino group.

Because of the expected acid lability of G-1-SA we only took into consideration reaction sequences which allow mild reaction conditions.

Two synthetic strategies are possible, the first *via* open chain and the second *via* a cyclic intermediate (pyroglutamic acid or derivatives).

First, selective reduction of the carbonic acid to a primary alcohol with diborane or borane-dimethylsulfide complex [19], and second, oxidation of the alcohol to an aldehyde function with pyridinium dichromate [20] or other approved reagents (Fig. 4). The reduction of **1** to the alcohol **6** was accomplished easily in 65% yield but the oxidation of **6** failed. Treatment of **6** with pyridinium dichromate yielded only overoxidized product **1** whereas **6** remained unchanged by treatment with pyridiniumchlorochromate [21]. Other reagents were not tried with regard to the acid lability of the protective groups.

The second synthetic strategy *via* pyroglutamic acid **8** [22], or its ethyl ester **9** as a cyclic intermediate could be an interesting alternative (Fig. 5).

The γ-carboxylic group is protected internally as a lactame function whereas the α-carboxylic group can

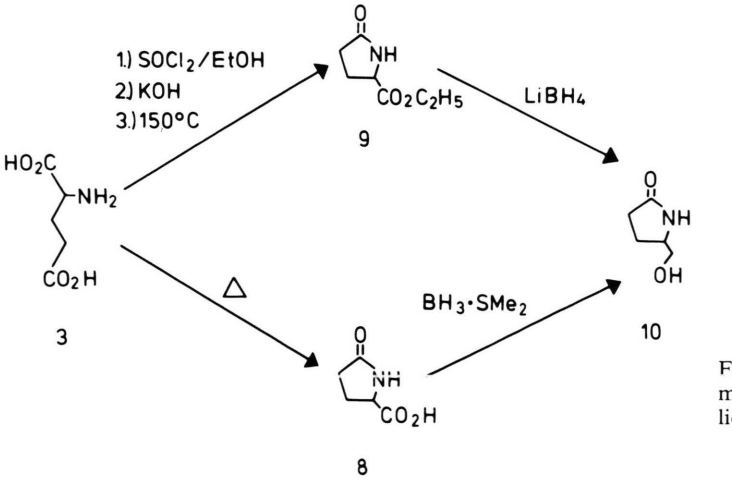


Fig. 5. Two different cyclic strategies for the formation of (*S*)-(+)-5-(hydroxymethyl)-2-pyrrolidinone (**10**), a possible precursor of G-1-SA.

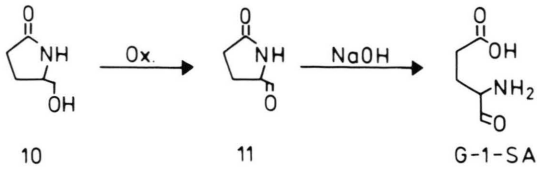


Fig. 6. Oxidation of (*S*)-(+)-5-(hydroxymethyl)-2-pyrrolidinone (**10**) to the corresponding aldehyde (**11**) by various oxidants and subsequent hydrolysis of the cyclic aldehyde by NaOH. G-1-SA could never be recovered.

be converted further. Pyroglutamic acid and its ethyl ester [23] are prepared easily and in good yield from glutamic acid. Conversion of **8** or **9** to the aldehyde **11** (Fig. 6) should be performed in two steps *via* alcohol **10** and its oxidation. Converting **11** to G-1-SA only requires saponification of the lactame group of **11**. Alcohol **10** was obtained by reduction of ester **9** with lithium borohydride in excellent yield [24], whereas reduction of pyroglutamic acid with borane-dimethylsulfide-complex yielded **10** in only 5% yield.

To oxidize **10** to aldehyde **11** we examined a variety of reagents:

- pyridiniumchromate [20];
- pyridiniumchlorochromate [21];
- chromic acid adsorbed on silica gel [25];
- chromium trioxide in hexamethylphosphoric triamide [26];
- chromium trioxide on anion exchange resin [27].

Although we observed a noticeable change of the reagents properties indicating the occurrence of oxidation reactions we could neither isolate the desired reaction product nor the starting material but only some polymer-like material. We therefore suppose that the expected aldehyde is not stable even under mild reaction conditions and polymerizes rapidly (see Ref. 14).

The stability of G-1-SA should be even smaller than that of **11** because of containing additionally a primary amino- and carbonic acid function.

The paper of Meisch and Maus [14] underlines the extreme tendency of G-1-SA to polymerization. The authors tried to synthesize G-1-SA by different and multistep procedures and came to initial products of G-1-SA. The attempt to remove their protecting groups never yielded G-1-SA but uncharacterized polymer products.

The results stated above are confirming the doubts of the chemical existence of G-1-SA we mentioned in the beginning. The findings exclude the possibility that Kannangara and Gough [9, 12] ever had free G-1-SA to their disposal as substrate for enzymatic tests. In contrary, as could be shown, the authors used glutamic acid instead of supposed G-1-SA as enzyme substrate. So the hypothesis of Kannangara and Gough [9, 12, 13] concerning the course of C5-pathway is remaining unproven.

## Experimental

### *General*

Nuclear magnetic resonance spectra were recorded on a Varian t-60 NMR spectrometer in $CDCl_3$ solution. Optical rotations were measured on a Perkin-Elmer-141 polarimeter.

Melting points were measured with a Büchi-Tottli apparatus and are not corrected.

Microanalysis were carried out by the analytical section of Fachbereich Chemie of the Philipps-Universität, Marburg.

### *Starting materials*

1. γ-Benzyl-L-glutamate (**4**) was prepared according to the procedure of Estrin *et al.* [15] in 38% yield, m.p. 172 °C. Anal. calc. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 61.03; H, 6.36; N, 5.99.
2. γ-Benzyl-N-carbobenzyloxy-L-glutamate (**1**) was prepared from **4** according to the procedure of Hanby *et al.* [16] in 42% yield, m.p. 74 °C (lit. [9], m.p. 76–78 °C). Anal. calc. for $C_{20}H_{21}NO_4$: C, 64.68; H, 5.70; N, 3.77. Found: C 64.60; H, 5.63; N, 3.84.
3. L-Pyroglutamic acid (**8**) was prepared from L-glutamic acid according to the procedure of Brenner and Rickenbacher [22] in 62% yield, m.p. 151–156 °C (lit. [15], m.p. 152–160 °C). $[\alpha]_D^{20} = -9.6°$ ($c = 5$; $H_2O$) (lit. [15], $[\alpha]_D^{20} = 9.9°$ ($c = 7.9$; $H_2O$).
4. (*S*)-(+)-5-Carbethoxy-2-pyrrolidone (**9**) was prepared from L-glutamic acid, ethanol and thionylchloride in 66% yield according to Silverman and Levy [23], b.p. 119 °C (0.1 mm) (lit. [16], b.p. 159–162 °C, (2 mm)). $^1H$ NMR, 60 MHz: 1.2 (t, $J = 7$ Hz, 3H); 1.9–2.6 (m, 4H); 3.9–4.4 (m, 1H); 4.1 (q, $J = 7$ Hz, 2H); 7.6 (broad signal, 1H).

### *Reaction of γ-benzyl-N-carbobenzyloxy-L-glutamate (**1**) with phosphorus pentachloride*

**1** was reacted with phosphorous pentachloride as reported by Kannangara and Gough [9, 12]. There-

fore a mixture of 3.5 g (10 mmol) of **1** and 2.35 g (11.3 mmol) of phosphorous pentachloride in 30 ml of dry ether was chilled to 0 °C and shaken until a clear solution was obtained. After decanting from some undissolved phosphorous pentachloride stirring was continued for 30 min at 0 °C. The yellow precipitate obtained was filtered by suction and washed with small amounts of petrol ether and carbon tetrachloride. 1.2 g of **5** (46% of theory) were obtained as colourless needles, m.p. 85–90 °C (lit. [18], m.p. 93–94 °C; lit. [9], m.p. 96–97 °C). $^1$H NMR, 60 MHz: 2.0–3.7 (m, 4H); 4.4 (t, $J$ = 7 Hz, 1H); 5.1 (s, 3H); 7.1–7.3 (m, 1H); 7.3 (s, 5H). Anal. calc. for $C_{13}H_{13}NO_5$: C, 59.31; H, 4.98; N, 5.32. Found: C, 58.78; H, 4.98; N, 5.31.

As to be expected from the work of Blout and Karlson [18] this reaction yielded γ-benzyl-N-carboxy-L-glutamic acid anhydride (**5**). Instead no γ-benzyl-N-carbobenzyloxy-L-glutamic acid chloride (**2**) which was proposed by Kannangara and Gough [9, 12] was formed. All other observations were in accordance with those of the cited authors.

*Catalytic hydrogenation of γ-benzyl-N-carboxy-L-glutamic acid anhydride (**5**)*

**5** was hydrogenated following the procedure of Kannangara and Gough [9, 12]. 1.18 g (4.5 mmol) of **5** being only sparingly soluble in diethyl ether, were dissolved in 6 ml of dry xylene. The reaction flask was flushed with nitrogen for five minutes, then 0.1 g of Pd-$BaSO_4$-catalyst were added. Hydrogenation was performed by bubbling hydrogen through the reaction mixture. After 30 min this mixture was filtered and the residue was washed three times with water. The combined aqueous filtrates were concentrated *in vacuo* to a 3 ml volume. To identify the products of this reaction the above aqueous solution was acidified and the products were separated on a Dowex 50 WX8 column as described [9, 12]. The only products that could be detected were glutamic acid and γ-benzyl-glutamate.

*Preparation of (S)-(−)-(n-carbobenzyloxy)-amino-5-hydroxybenzyl-pentanoate (**6**)*

To an ice-cooled solution of 4.0 g (10.7 mmol) of **1** in 30 ml of dry ether 1.5 ml of 10 molar borane-dimethylsulfide-adduct, which was dissolved in 10 ml of dry ether, were added dropwise within 5 min. After stirring for 1 h at room temperature the reaction mixture was hydrolyzed by carefully adding 10 ml of glycerine-water (1:3, v/v) followed by a small amount of $NaHCO_3$. Then the phases were separated and the aqueous phase was extracted with ether. The combined etherous phases were dried over $MgSO_4$ and concentrated *in vacuo* yielding 3 g of a colourless oil, which was crystallized from $CCl_4$ and dried over $P_4O_{10}$. 2.49 g (65% of theory) of pure **6** were obtained. M.p. 70.5 °C; $[\alpha]_D^{20} = -16.5°$ ($c$ = 2.5; $CHCl_3$); $^1$H NMR, 60 MHz: 1.5–2.3 (m, 5H); 3.3–3.4 (m, 2H); 4.4–4.5 (m, 2H); 4.85 (s, 2H); 4.9 (s, 2H); 7.3 (s, 10H). Anal. calc. for $C_{20}H_{23}NO_5$: C, 67.21; H, 6.49; N, 3.92. Found: C, 66.85; H, 6.47; N, 4.03.

*Preparation of (S)-(+)-5-(hydroxymethyl)-2-pyrrolidinone (**10**)*

a) 12.9 g (0.1 mol) of L-pyroglutamic acid were suspended in 50 ml of dry ether. To this 13 ml of a 10 molar borane-dimethylsulfide-adduct were added at 0 °C, which were dissolved in 20 ml of dry ether. After stirring for 6 h at room temperature the reaction mixture was hydrolyzed by carefully adding 50 ml of glycerine-water (1:3, v/v). Then the phases were separated and the aqueous phase was extracted with ether. The combined etherous layers were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. High vacuum distillation yielded 5.8 g (5% of theory) of **10** as a yellowish oil which crystallized on cooling. M.p. 66–70 °C (lit. [23], m.p. 66–68 °C; lit. [24], m.p. 78 °C). $[\alpha]_D^{20} = +22.8°$ ($c$ = 10; ethanol) (lit. [23], $[\alpha]_D^{20} = +29°$; lit. [24], $[\alpha]_D^{20} = +32.4°$). $^1$H NMR, 60 MHz: 1.5–2.6 (m, 4H); 3.3–4.0 (m, 3H); 5.0 (broad signal, 1H); 7.6 (broad signal, 1H).

b) **10** was prepared by reduction of (*S*)-(+)-5-carbethoxy-2-pyrrolidinone (**9**) with $LiBH_4$ in THF in 96% yield according to the procedure of Faber and Wiegrabe [24]. M.p. 60–63 °C. $^1$H NMR as above.

*Attempted preparation of (S)-5-carboaldehyde-2-pyrrolidinone (**11**) via oxidation of (S)-(+)-5-(hydroxymethyl)-2-pyrrolidinone (**10**)*

a) 3.43 g (30 mmol) **10** in 120 ml dry $CH_2Cl_2$ were added to an ice-cooled stirred suspension of 22.6 g (60 mmol) pyridinium dichromate [20] in 80 ml dry $CH_2Cl_2$. After 2 h the suspension turned into a solid brown lump. It was diluted with ether and decanted. The lump was ground and extracted

with ether. The combined solutions were filtered through silica gel and concentrated *in vacuo* yielding a viscous brown tar which could not be characterized.

b) To 11.9 g (150 mmol) pyridine in 160 ml $CH_2Cl_2$ 7.5 g (75 mmol) $CrO_3$ were added at room temperature. After 15 min the solution was cooled to 0 °C and 1.15 g (10 mmol) of **10**, dissolved in 40 ml $CH_2Cl_2$ were added and stirred for 40 min. Then 36 g (0.3 mmol) powdered $NaHSO_4$ were added and stirring continued for additional 30 min. After filtering by suction and drying with $Na_2SO_4$ the solvent was evaporated yielding small amounts of viscous non-characterizable substance.

c) To a suspension of 15 g of a reagent prepared from $CrO_3$ and silica gel [25] in 25 ml dry ether 0.58 g (5 mmol) **10** in 25 ml dry THF were added at room temperature and stirred for 1 h. After filtration and washing the solid with ether, the filtrate was evaporated to dryness yielding a small amount of dark viscous residue.

d) 2 g $CrO_3$ were added to 6 ml dry HMPT in small portions. After 1 h at room temperature 1.16 g (10 mmol) **10** in 3 ml HMPT were added and stirred for 6 h. The reaction mixture was poured into iced water and extracted with ether. The combined extracts were washed with 5% NaOH and dried over anhydrous $Na_2SO_4$. After evaporation of the solvent a small amount of viscous brown substance remained.

e) Some experiments with $CrO_3$ on Dowex according to Cainelli *et al.* [27] had the same results.

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