Production of L-Tryptophan from γ-Glutamaldehydic Acid

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Synopsis. Labile γ-glutamaldehydic acid (**1**) was first converted to tryptophan via its phenylhydrazone; no racemization occurred during the reaction. The stability of **1** was shown to be remarkably enhanced in the presence of additives.

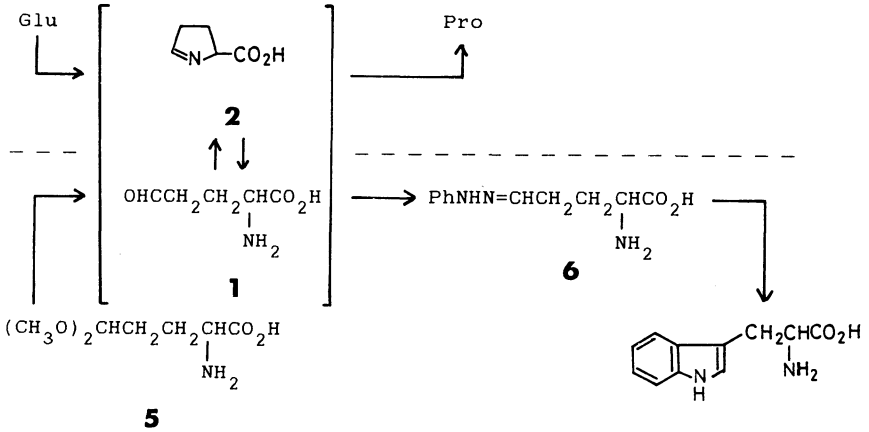
The production of L-tryptophan (L-Trp) has been extensively investigated after its positive effects as a feed additive became recognized.[1] We focused our attention on obtaining L-Trp by a combination of a fermentation and chemical synthesis. Practical synthetic methods for the production of L-Trp generally require an optical resolution of the racemic form, followed by the racemization of D-Trp.[2] Our process dose not necessitate these steps since optically active starting material prepared by fermentation is used.

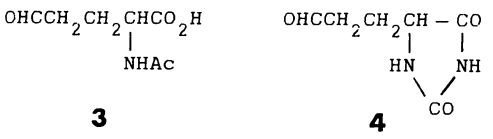
γ-Glutamaldehydic acid (GAA, **1**) and its cyclic imine form, 3,4-dihydro-2*H*-pyrrole-2-carboxylic acid, were known to be labile intermediates in the biosynthesis of proline from glutamic acid.[3-5] GAA (**1**) was likely to be a suitable starting material of the nonprotected L-Trp synthesis by a Fischer indole formation. GAA (**1**), however, has neither been isolated in a pure form nor has it been used as a useful synthetic intermediate, probably because of its instability. The stability of **1** depended on its concentration; 30% of **1** in a 50-mM neutral solution (1 M=1 mol dm^{-3}) was reported to decompose within 1 hour.[6] On the other hand, none of these changes were observed in a 1-mM solution.[6] In the course of our practical research toward the establishment of a production method for L-Trp, 1) the possibility of chemical conversion of **1** to Trp, 2) the stereochemistry during the reaction, and 3) the way for preservation of **1** in neutral solution, were investigated.

In order to mimic the combination of biosynthesis of **1** and chemical conversion of it to Trp, GAA (**1**) was conveniently generated by the acidic hydrolysis of GAA dimethyl acetal (**5**), because L-**1** was not currently available by a fermentative production.[8] Since the phenylhydrazone (**6**) did not quantitatively precipitate due to its solubility, phenylhydrazone formation and indole synthesis were carried out consecutively. *N*-Ac-GAA (**3**) and GAA-hydantoin (**4**) were reported to afford the corresponding protected Trp in good yield under mild reaction conditions: [0.1 M HCl, reflux, 1 equiv of phenylhydrazine].[9] Meanwhile, for the case of **1**, Trp was provided in poor yield under the same conditions as mentioned above. The yield was shown to depend on the acidity and the amount of phenylhydrazine (Table 1). When 15 equiv of phenylhydrzine and 0.45 M (1 M=1 mol dm^{-3}) sulfuric acid was used, the result was remarkably improved (85% yield). GAA (**1**) in a

Table 1. Synthesis of Trp from **5** under Various Conditions

$PhNHNH_2 \cdot HCl$ (equiv to **1**)	H_2SO_4/M	Yield/%
1.0	0.05	17
1.0	0.45	27
7.0	0.45	62
15.0	0.45	85





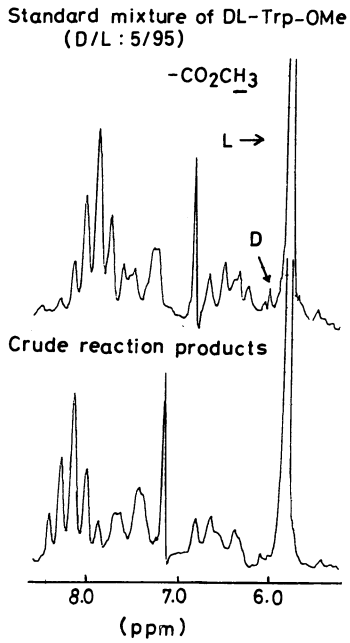


Fig. 1. ^{1}H NMR spectrum of standard mixture of DL-Trp-OMe (D/L: 5/95) and crude reaction products after adding chiral shift reagent.

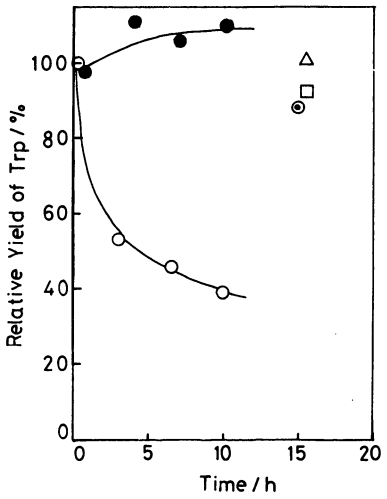


Fig. 2. Stabilizing effect of additives to solution of **1**. ● $PhNHNH_2 \cdot HCl$, △ $NaHSO_3$, □ $NH_2OH \cdot HCl$, ⊙ $H_2NCONHNH_2$, ○ none.

neutral solution provided the same results (85% yield) under optimum reaction conditions.

The previously mentioned successful observation suggested that L-**1** in a fermentation broth was able to be effectively converted to L-Trp. We therefore investigated whether isomerization took place during the course of the reaction. Optically active **5** was prepared by enzymatic deacylation of racemic *N*-acetyl-**5**. Compound L-**5** was subjected to the aforementioned Trp synthesis followed by esterification. The optical purity of the obtained L-Trp methyl ester (L-Trp-OMe) was examined by ^{1}H NMR using a chiral shift reagent. The ^{1}H NMR spectra of the reaction products were compared to the spectra of a mixture (95:5) of L- and D-Trp-OMe in the presence of tris[3-(trifluoromethylhydroxymethylene)-*d*-camphorato]europium(III).[10] Since no signal corresponding to D-Trp-OMe was detected in the reaction product (Fig. 1), it was concluded that isomerization did not occur during the reactions.

Since an accumulation of **1** in the fermentation broth with a high concentration is desired in order to produce L-Trp on an industrial scale, we investigated the enhancement of the stability of **1** in a neutral environment.

When a 230-mM solution of **1** (pH 7) was left at 31 °C for several hours, followed by treatment with phenylhydrazine in acidic solution, Trp formation at an appropriate time decreased linearly with a time (Fig. 2). Meanwhile, reagents which generally react with aldehydes such as phenylhydrazine, sodium hydrogensulfite, hydroxylamine, and semicarbazide were added to a neutral solution of **1** before left standing, in order to form a stable adduct. As expected, no substantial decrease in Trp formation was observed, even after 15 hours. This meant that **1** could be preserved under standard fermentation conditions (pH 7, 31 °C). A stable form was exhibited to have the same chemical reactivity as **1** from the Trp synthesis.

These results strongly indicate the possibility of accumulating L-**1** by fermentation without decomposition and conversion to L-Trp. This process is highly straightforward and practically attractive. Further, progress in the fermentative production of L-**1** and its conversion to L-Trp will be reproted elsewhere.[8]

Experimental

High performance liquid chromatography (HPLC) was obtained with a Hitachi 635 fitted with a 500 mm×2.1 mm column containing Hitachi cation resin #2610. The eluent was a phosphate buffer (0.1 M KH_2PO_4: 0.1 M Na_2HPO_4= 16:1) at a flow rate of 0.5 ml min^{-1}. The temperature was 52 °C and detection was at 274 nm. IR and NMR spectra were recorded on a JASCO Infrared Spectrometer Model IR-S and Varian NMR Spectrometer XL-100A, respectively. The optical rotation was determined on a JASCO Digital Polarimeter DIP-140. Melting points are uncorrected. Racemic GAA dimethyl acetal (**5**) was prepared according to the procedure reported by Okuda.[7] Aminoacylase derived from *Aspergillus oryzae* was purchased from Amano Pharmaceutical Co.

Trp (general procedure). **Method A.** To the solution of phenylhydrazine hydrochloride (2.46 g, 17.0 mmol) in 0.9 M sulfuric acid (6 ml) was added **5** (201 mg, 1.1 mmol) in water (6 ml) dropwise under reflux. The reaction was carried out for 1 hour and analyzed by HPLC (85% yield). **Method B.** To a cooled solution of 1 M hydrochloric acid (10 ml) in an ice bath was added **5** (201 mg, 1.1 mmol). The mixture was stirred for 30 minutes and neutralized to pH 7 by adding 1 M aq. sodium hydroxide solution. This solution was added to a refluxing solution of phenylhydrazine hydrochloride (2.45 g, 17.0 mmol) in 0.9 M sulfuric acid (15 ml). The reaction mixture was refluxed for 1 hour and analyzed by HPLC (85% yield).

L-γ-Glutamaldehydic Acid Dimethyl Acetal (L-5). To a solution of **5** (3.39 g, 1.9 mmol) in 0.44 M aq. sodium hydroxide (50 ml) was added acetic anhydride (2.5 g,

25 mmol) while keeping the pH at 9 by the occasional addition of aq. sodium hydroxide at room temperature. The mixture was stirred for 1 hour and lyophilized after the pH was adjusted to 7. The residue was dissolved in water (80 ml) and 10 mM cobalt(II) chloride solution (4 ml) and amino-acylase (198.5 mg) were added. The enzymic reaction was carried out for 92 hours at 37 °C and the pH was kept in the range of 7.5 to 8.0. Inorganic salts were removed by adding a cation-exchange resin (IRC-50) and an anion-exchange resin (IR-45). The solvent was evaporated under reduced pressure and the residue (1.2 g) in water (400 ml) was charged to a sirotherm resin (supplied by Japan Sirotherm Co., Inc.) (80 ml) in column in order to remove any unreacted *N*-acetylated **3**. The column was washed with water (250 ml) at 0 °C and then eluted with water (300 ml) at 80 °C. The eluent was lyophilized and the residue was recrystallized from water (1 ml) and ethanol (1 ml) and diethyl ether (2 ml) to afford 93.3 mg (0.53 mmol) of L-**5**. $[\alpha]_D^{25}$ −1.07 (*c* 0.93, water).

L-Trp-OMe. L-**5** (93.3 mg, 0.3 mmol) was converted to L-Trp as previously described. The resultant solution was adjusted to pH 9.5 and washed with benzene and diethyl ether to remove phenylhydrazine. The aqueous phase was neutralized and concentrated to dryness. The residue was dissolved in methanol (20 ml) and esterified by bubbling HCl gas for 1.5 hours at room temperature. The solvent was removed and the residue, dissolved in chloroform, was washed with aq. sodium hydrogencarbonate, water and dried to give L-Trp-OMe (46.5 mg, 0.21 mmol).

Determination of Optical Purity of L-Trp-OMe. The Kainosho method was used.[10]

Stability of γ-Glutamaldehydic Acid (1). GAA dimethyl acetal (**5**, 280 mg, 1.58 mmol) was treated with 1 M HCl (7 ml) at 5 °C overnight. The mixture was allowed to warm to room temperature and then neutralized by adding aq. sodium hydroxide. To this solution of **1** was added equimolar amounts of reagents ($PhNHNH_2 \cdot HCl$, $NaHSO_3$, $NH_2OH \cdot HCl$, $H_2NCONHNH_2 \cdot HCl$). The pH was then adjusted to 7 and the volume adjusted to 10 ml. The mixture was left at 31 °C for an appropriate time and then converted to tryptophan using optimum reaction conditions. The yield of tryptophan was compared to the yield when nothing was added to a solution of **1**.

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