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THE ^{15}N LABELLED L-GLUTAMIC ACID: EXPERIMENTAL AND COMPUTATIONAL NMR STUDIES

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^{15}N labelled L-glutamic acid is synthesized by the reductive amination of α-ketoglutarate, in the presence of glutamate dehydrogenase (GDH), nicotinamide adenine dinucleotide (NADH) as cofactor and $^{15}NH_4Cl$ as source of isotope ^{15}N. The reaction mixture includes glucose dehydrogenase (GlcDH) and glucose, as electron donor, for NADH regeneration, too. We are also interested in the theoretical calculation of NMR parameters for this compound; the ^{13}C chemical shifts and coupling constants were calculated by B3LYP (hybrid Becke 3 Lee Yang Parr) density functional method with 6-311+G(d,p) basis set. For chemical shifts and coupling constants prediction the GIAO (Gauge-Induced Atomic Orbital) approximation and the keyword NMR=spinspin respectively were used. Comparison between the experimental and theoretical results indicates that DFT/B3LYP/6-311+G(d,p) method is in good agreement with the experimental data.

INTRODUCTION

Existent studies in the fields of biochemistry, geochemistry, medicine, pharmacology, etc. have shown the utility of stable isotopes labelled amino acids synthesis. This interest is due to its wide range of applications in, for example, determination of biological macromolecules (proteins, nucleic acids) structure and functions by nuclear magnetic resonance (NMR) spectroscopy,[1] enzyme mechanisms determination,[2] quantification of drugs and metabolites in biological fluids and pharmacokinetic parameters determination.[3,4]

L-glutamic acid is one of the twenty proteinogenic amino acids and also an important brain neurotransmitter. Its occurrence in proteins is about 6.3%[5] and dysfunctions in glutamate levels are involved in acute mania and several neurodegenerative disorders.[6,7] It contains an acidic side chain and it is usually called glutamate because at physiological pH its side chain is negatively charged.

NMR spectroscopy is currently used for structure and function determination of biological macromolecules.[8] *In vivo* NMR spectroscopy (MRS), especially ^{13}C and ^{1}H MRS, is a valuable method for the study of neurodegenerative diseases and metabolism in animal models or even humans.[9,10] Chemical shifts are recognized as an important part of the information contained in NMR spectra. They are useful for structural interpretation due to their sensitivity to conformational variations. The combined use of NMR and computer simulation methods offers a powerful way to interpret and predict the structure of large biomolecules.[11]

L-amino acids, like building blocks of proteins, were intensively investigated by experimental and theoretical approaches; for L-glutamic acid, a lot of density functional theory (DFT) investigations: gas-phase acidity and basicity, proton affinity, ^{13}C NMR and structural studies on the hydration in solution were done.[12-17]

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In the present paper we describe the synthesis of ^{15}N labelled L-glutamic acid by reductive amination of α-ketoglutarate, in the presence of glutamate dehydrogenase (GDH), nicotinamide adenine dinucleotide (NADH) as cofactor and $^{15}NH_4Cl$ as source of isotope ^{15}N. The structural characterization of the labelled amino acid was done by ^{15}N, ^{1}H and ^{13}C NMR spectroscopy. Determination of the ^{15}N enrichment of amino acid was realized by ^{13}C NMR spectroscopy and suppose quantitative analysis of ^{13}C in the region 48-58 ppm. In order to optimize and calculate NMR parameters with reasonable computational costs (quantitative results accuracy, CPU time and resource requirements) a theoretical study was realized using Gaussian 03 package[18] and the graphical interface Gabedit 2.2.12.[19] We focused on two levels of theory, HF (Hartree Fock) and DFT (Density Functional Theory), and polarised (6-31G(d), 6-311G(d,p)) or diffuse 6-311+G(d,p)) basis sets.

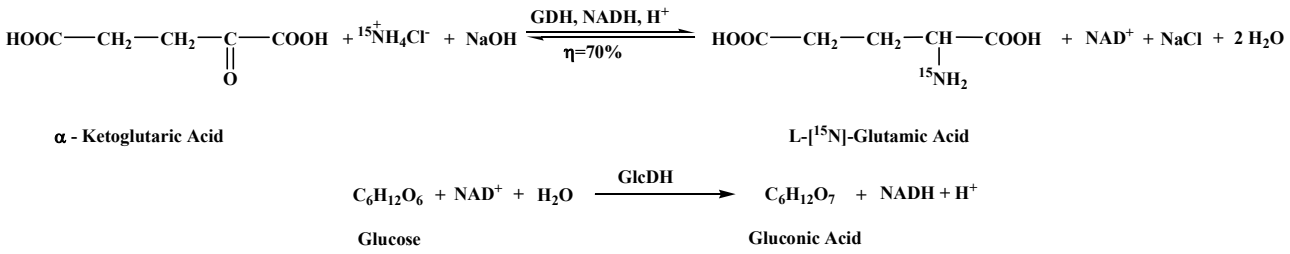
RESULTS AND DISCUSSION

1. Enzymatic synthesis and experimental investigation of L-[^{15}N]-glutamic acid

The enzymatic synthesis of ^{15}N isotopically labelled L-glutamic acid developed by us is shown in Scheme 1.

The reductive amination of α-ketoglutaric acid by the Nicotinamide Adenine Dinucleotide (NADH) to form L-glutamic acid is catalyzed by the enzyme Glutamate Dehydrogenase (GDH) from bovine liver. The cofactor NADH regeneration is realized by the conversion of glucose into gluconic acid by the enzyme Glucose Dehydrogenase (GlcDH) from *Bacillus subtilis*. The two enzymes present the following properties: bovine glutamate dehydrogenase (GDH) has optimal activity at 30°C and pH between 7 and 9,[20] and glucose dehydrogenase from *Bacillus subtilis* at temperature between 23-37°C and pH 8.[21] Under these circumstances, the reaction is conducted at 30°C and pH 8 (the pH is maintained at 8 by adding NaOH). The incorporation of ^{15}N stable isotope into L-amino acid is realized by the presence in the reaction mixture of ammonium chloride 99% at. ^{15}N labelled. This method allows us to obtain a 99% at. ^{15}N enrichment of amino acid (no isotope dilution) and can be applied at large scale 1-10 grams; the synthesis is also stereoselective and regioselective. Even the yield is no so good (only 70%), the fact that no isotope dilution was found makes this synthesis attractive for ^{15}N enrichment of L-glutamic acid. A similar procedure was used by Zintel and co-workers; in their case, the labelled amino acid is recovered in yield of 47% and, also, a significant isotope dilution was found due to the enzymes impurities.[22]

The determination of ^{15}N enrichment of amino acid was realized by ^{13}C NMR spectroscopy and suppose quantitative analysis of ^{13}CH (C-2, Fig. 1) in the region 48-58 ppm. In L-[^{15}N]-glutamic acid, the ^{15}N nucleus with a spin ½ couples with nearby carbon, CH to give a coupling constant, $^{1}J(^{15}NH_2\text{-}CH)$= 5.7 Hz. This situation doesn't occur in unlabelled L-amino acid.



Where: NAD^+:Nicotinamide Adenine Dinucleotide (oxidated form); NADH, H^+: Nicotinamide Adenine Dinucleotida (reduced form)
GDH: Glutamate Dehydrogenase; GlcDH: Glucose Dehydrogenase

Scheme 1 – Enzymatic synthesis of L-[^{15}N]-glutamic acid.

Isotopic content was calculated by formula:

$$\%\ \text{at. } ^{15}N \text{ amino acid} = 100 - \frac{\text{Area of } \delta\ (53.87\ \text{ppm}) - \text{Area of } \delta\ (53.83\ \text{ppm})}{\text{Area of } \delta\ (53.87\ \text{ppm}) + \text{Area of } \delta\ (53.83\ \text{ppm})} \times 100$$

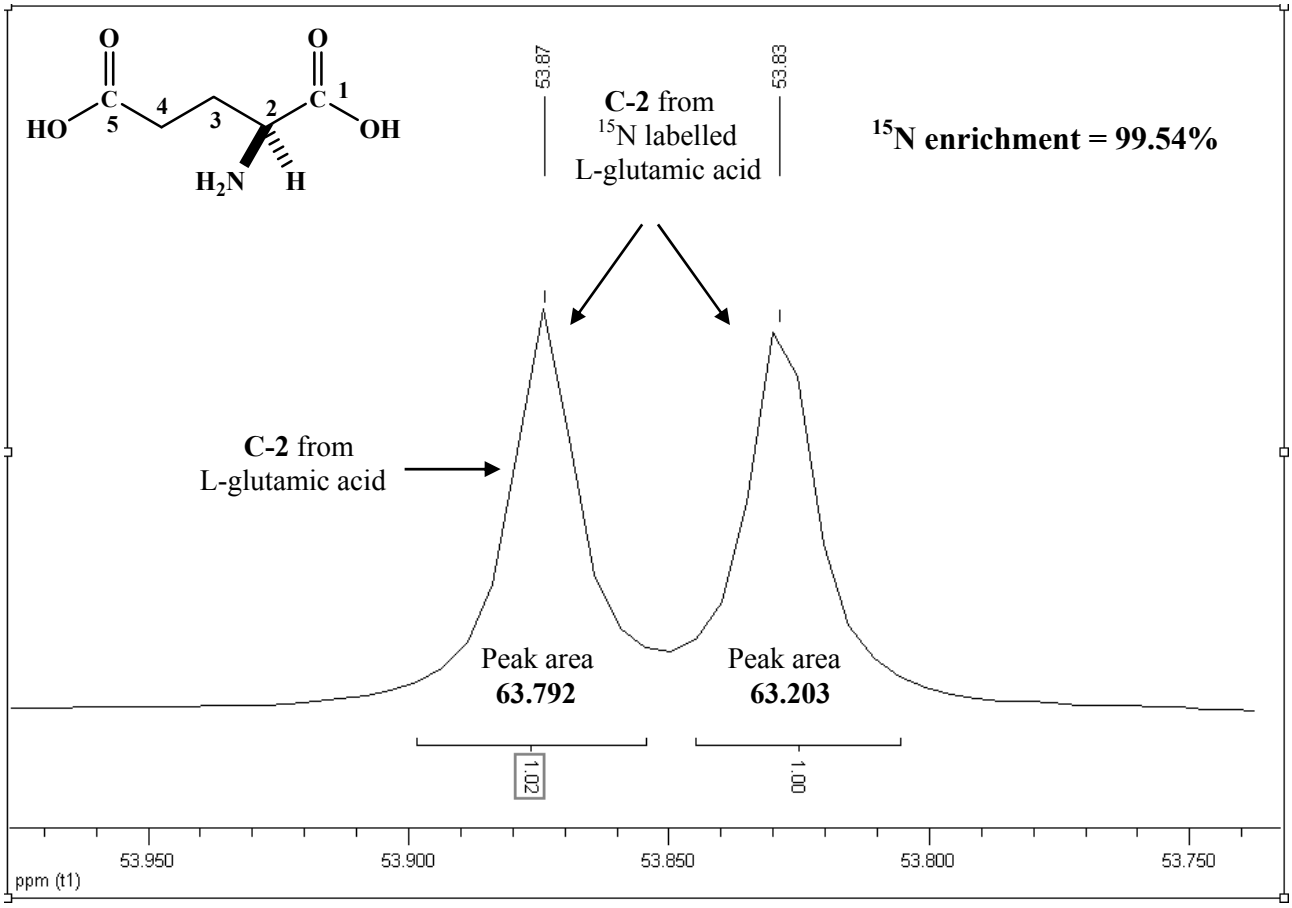


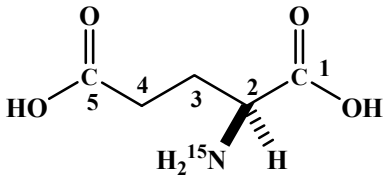
Fig. 1 – The CH (C-2) region of ^{13}C NMR spectra for L-glutamic acid (125 MHz, D_2O).

The result was compared to a conventional method: gas chromatographic-mass spectroscopy (GC/MS) that involves the conversion of the amino acid to the *tert*-buthyldimethylsilyl (TBDMS) derivative. In both cases, the isotope content of L-[^{15}N]-glutamic acid was found to be over 99% at. ^{15}N.[23]

Fig. 2 shows the ^{15}N CP/MAS of L-[^{15}N]-glutamic acid. The CP/MAS experiment was performed at 40.54 MHz for ^{15}N on a Bruker Avance 400 spectrometer; ^{15}N chemical shift, relative to NH_4^+ resonance at 0 ppm, is 12.6 ppm, Figure 2(a). The ^{1}H and ^{13}C NMR spectra, Fig. 2(b), (c), were recorded in D_2O, on a Bruker Avance 500 NMR spectrometer with TMS as internal standard, at 500 and 125 MHz respectively.

In the ^{1}H NMR spectrum, Fig. 2(b), we find three multiplets; of the three signals which can be seen, the triplet at δ=3.74 ppm can be assigned to the proton on C-2; the protons on C-3 are coupled both to the protons on C-2 and to the protons on C-4, so the multiplet at δ=2.07 ppm is assigned to the two protons on C-3 and the peak at δ=2.48 ppm to the protons on C-4.

The ^{13}C NMR spectrum, Fig. 2(c), shows the coupling between $^{15}NH_2$ and C-2, $^1J(^{15}NH_2\text{-}CH)$= 5.7 Hz. The ^{13}C chemical shielding for L-[^{15}N]-glutamic acid are: C-5 at 177.0 ppm, C-1 at 173.8 ppm, C-2 at 53.7 ppm ($^1J(^{15}NH_2\text{-}CH)$= 5.7 Hz), C-4 at 29.9 ppm and C-3 at 25.4 ppm.



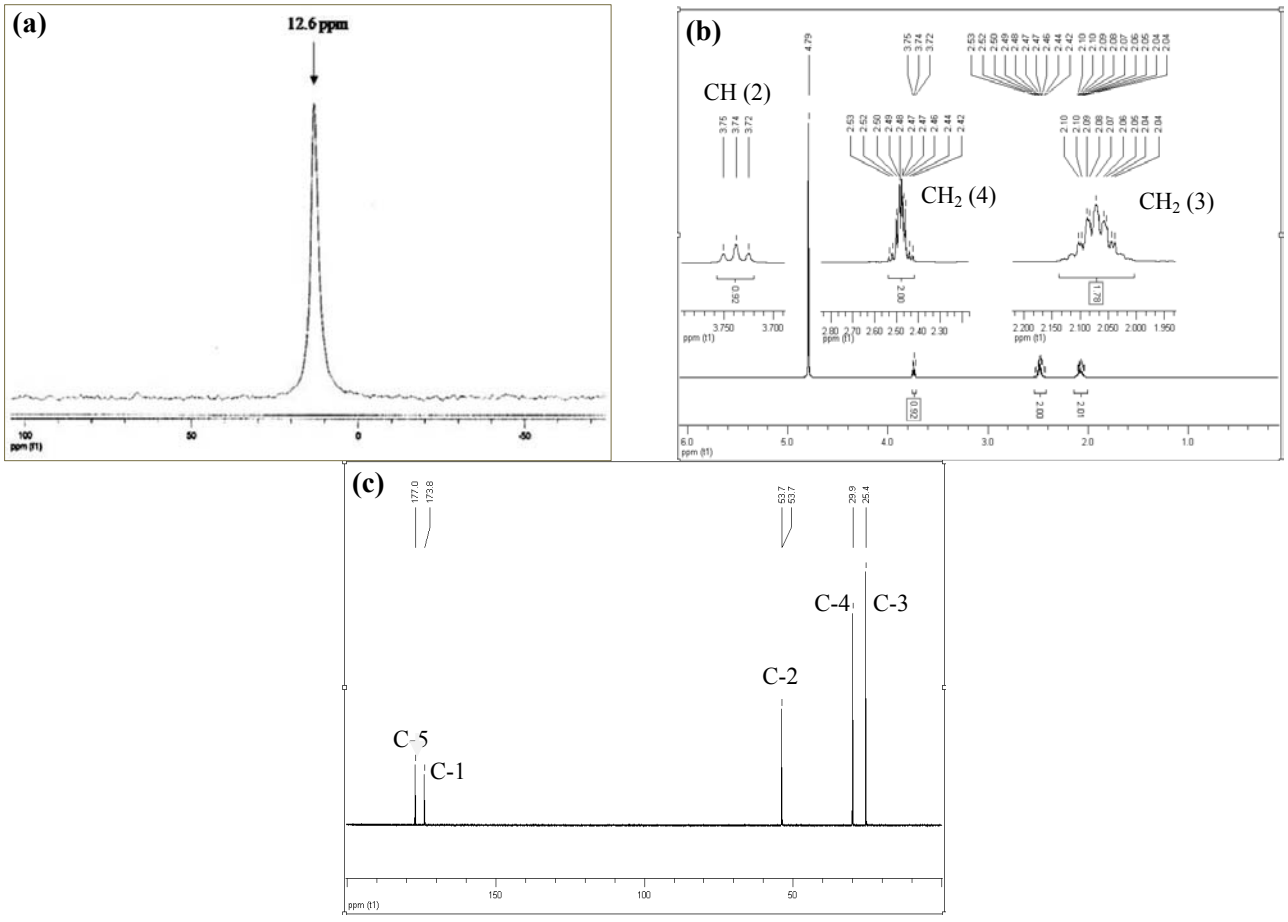


Fig. 2 – The L-[^{15}N]-glutamic acid NMR spectra: (a) ^{15}N CP/MAS at 40.54 MHz, (b) ^{1}H NMR in D_2O at 500 MHz, (c) ^{13}C NMR in D_2O at 125 MHz.

2. Theoretical calculation

Amino acids can adopt different conformations according to the environment; they can exist as zwitterions in solids, polar solutions (water) and as neutral (non-zwitterionic) form in gas phase.[24,25] Sun reported for neutral D,L-glutamic acid, in gas-phase, 21 local minima on its potential energy surface (PES).[15] We optimized, without any constraints, the lowest free energy structure reported by Sun as well as some additional structures found by us for L-glutamic acid, by HF (Hartree-Fock) and DFT/B3LYP methods with 6-31G(d), 6-311G(d,p) and 6-311+G(d,p) basis sets. For neutral zwitterionic form of L-glutamic acid, the effect of the solvent (water) is simulated by using the polarized continuum model (PCM) within the self-consistent reaction field (SCRF) method with the dielectric constant, Eps= 78.39.

The calculated energies (E, Hartree) are listed in Table 1. The best results are obtained by using density functional theory: B3LYP level and 6-311+G(d,p) as basis set. Accordingly, the DFT/B3LYP method was addopted for the following NMR computation.

The B3LYP/6-311+G(d,p) optimized structures of L-glutamic acid (a) in gas-phase, (b) in solution (water) are shown in Fig. 3. The intramolecular O=C-OH...NH_2, HNH...O=C-OH and COOH hydrogen bonds were also relieved.

Tabel 1

Calculated Energies (Hartree) of the neutral and the neutral zwitterion form of L-glutamic acid

L-glutamic acid	**Methods and Energies (Hartree)**					
	HF			**DFT/B3LYP**		
	6-31G(d)	6-311G(d,p)	6-311 +G(d,p)	6-31G(d)	6-311G(d,p)	**6-311 +G(d,p)**
Neutral form (Gas-phase)	-548.5167	-548.6747	-548.6858	-551.6232	-551.7979	**-551.8139**
Neutral zwitterion form (Water)	-548.5486	-548.7043	-548.7218	-551.6532	-551.8271	**-551.8500**

The ^{13}C absolute shieldings of L-glutamic acid were calculated over the fully optimized geometry within the GIAO (gauge-induced atomic orbital) aproximation.[26] The coupling constants were obtained by using the keyword NMR=spinspin. Because the quantum calculations give absolute chemical shielding values, σ_{ii}, we must convert them into chemical shifts relative to the reference, δ_{ii}, by using the formula: $\delta_{ii} = \sigma_{reference} - \sigma_{ii}$ **(ppm)**. In this case, the reference (TMS-tetramethylsilane) shieldings were calculated in advance at the same theoretical level as for L-glutamic acid. The calculated chemical shieldings and $^1J(^{15}\text{N-CH})$ coupling constant are listed in Table 2.

The ^{13}C simulated NMR spectra of fully optimized neutral and neutral zwitterionic form of L-glutamic acid by GIAO method at B3LYP/6-311+G(d,p) level of theory are presented in Figs. 4 and 5. With the exception of carboxyl carbon nuclei C-7 (in gas phase) and C-1, C-7 (in solution), a good correlation is observed between experimental and calculated data. The magnitude of the contributions to the spin-spin coupling constant (Fermi-contact, diamagnetic, and paramagnetic spin−orbit contributions) and total nuclear spin-spin coupling *J* (Hz) were determined. The simulated $^1J(^{15}\text{N-CH})$ values were different from the experimental data and for this calculation the problem of basis set remains open to further investigation.

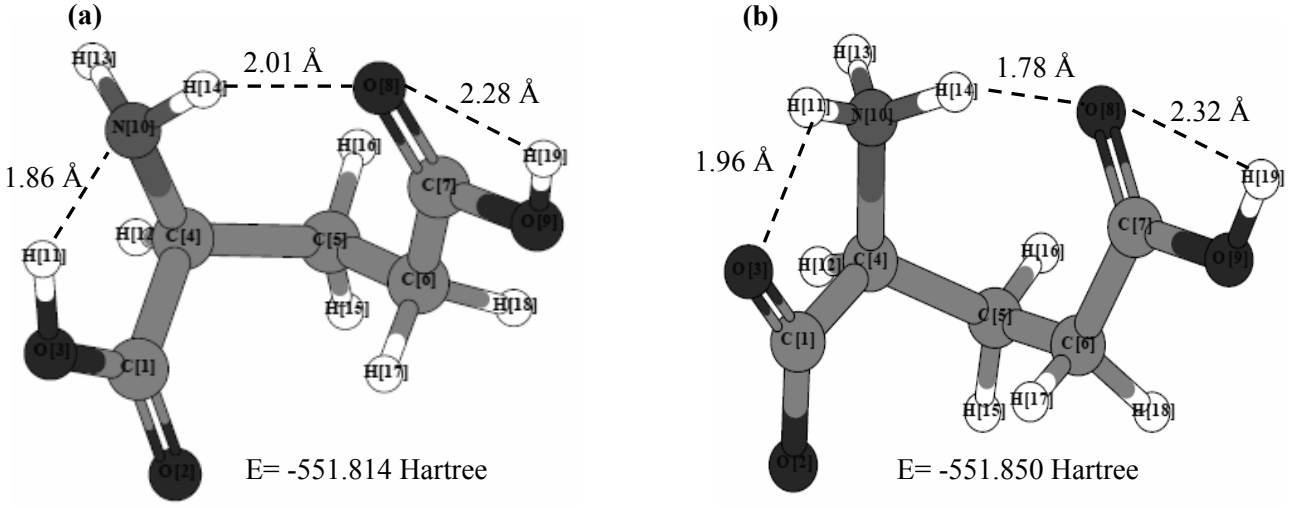


Fig. 3 – The optimized structures of L-glutamic acid by B3LYP/6-311+G(d,p) in (a) neutral form (gas-phase) and (b) neutral zwitterionic form (water).

Table 2

Experimental and theoretical ^{13}C NMR relative chemical shielding, δ, ppm and $^{1}J(^{15}N\text{-}CH)$, Hz data of L-glutamic acid

L-glutamic acid	**^{13}C Chemical shifts (ppm)**					**B3LYP/6-311+G(d,p)/ NMR=spinspin**
	B3LYP/6-311+G(d,p)/NMR=GIAO					
	C-5	C-6	C-4	C-1	C-7	$^{1}J(^{15}N\text{-}CH)$ (Hz)
Neutral form (Gas-phase)	26.1	28.1	55.0	173.3	179.96	3.0
Neutral zwitterion form (Water)	24.2	29.7	54.9	164.7	185.7	1.9
Experimental Bruker Avance 500MHz	25.4	29.9	53.75 53.7	173.76	176.99	6.3

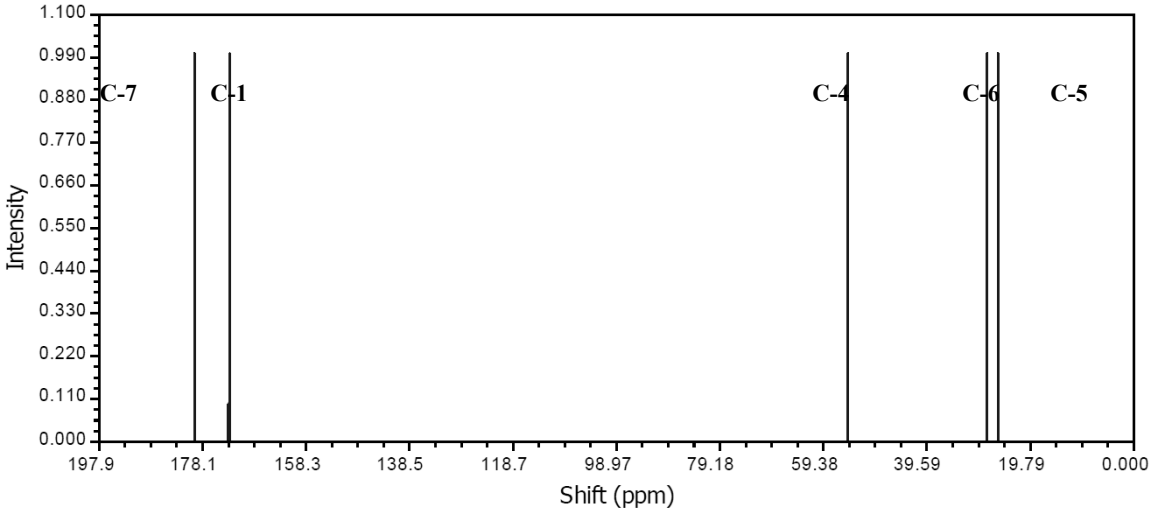


Fig. 4 – ^{13}C simulated NMR spectrum of optimized neutral L-glutamic acid by GIAO method at B3LYP/6-311+G(d,p) level of theory.

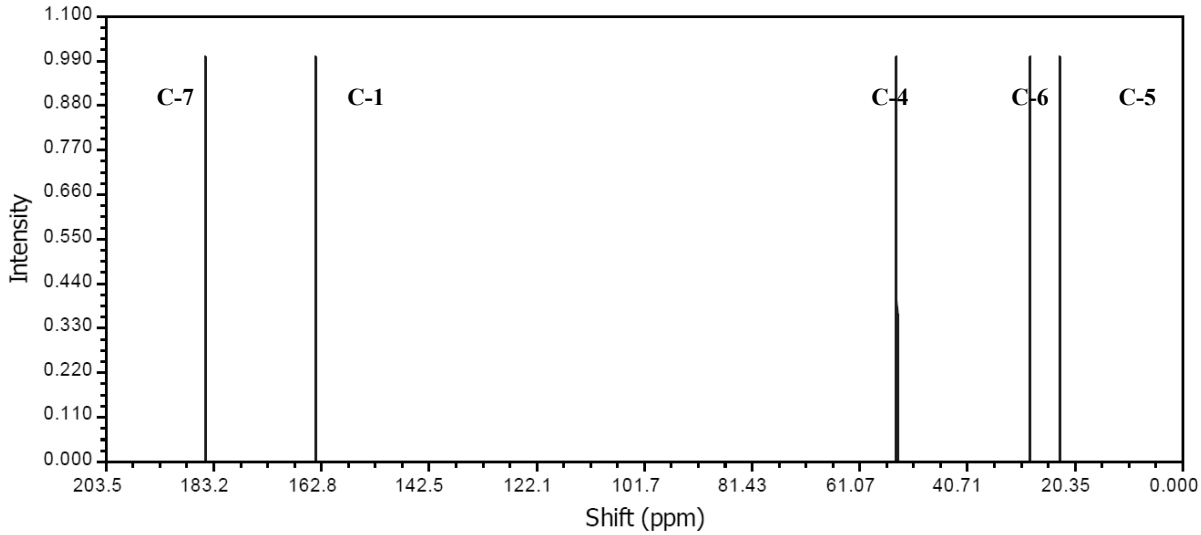


Fig. 5 – ^{13}C simulated NMR spectrum of optimized neutral zwitterionic L-glutamic acid by GIAO method at B3LYP/6-311+G(d,p) level of theory.

One can also note that in both spectra, the chemical shifts of COOH (C-7) are deshielded: Δδ= -2.97 ppm for neutral L-glutamic acid and Δδ= -8.71 ppm for neutral zwitterionic form (the problem is more evident in the latter compound). But, for the other carboxyl group, C-1, we observe a shielding effect: unsignificant (Δδ= +0.46 ppm) for the neutral form and high (Δδ= +9.06 ppm) for the zwitterionic form. This feature is probably related to the solvent effect and more solvation models should be investigated.

EXPERIMENTAL PART

The synthesis of [^{15}N]-labelled L-glutamic acid was performed, according to a method previously established by our group,[23] as follows: 40 mmol glucose, 8 mmol α-ketoglutarate (Na-salt), and 8 mmol [^{15}N] ammonium chloride (~99 atom % ^{15}N) were dissolved in 70 mL of bidistilled water maintained at 30°C. The pH was adjusted to 8.0 with 1 N NaOH. Then, 0.5 mmol NADH, GDH (100 U) and GlcDH (110 U) were added. Under these conditions the formation of [^{15}N]-labelled L-glutamic acid was accompanied by the release of gluconic acid and the decrease of pH accordingly. By adding small volumes of 1 N NaOH the pH was restored to 8.0-8.2. After 2-3 hours, the reaction was finished and no further change in pH was observed. Finally, the reaction mixture was heated to 85°C for 15 minutes. After cooling down the reaction mixture, the denaturated proteins were removed by filtration or centrifugation. The precipitate was filtered and the solution was concentrated under vacuum to a small volume, and then 1 N HCl solution was added until pH 3.2 (the isoelectrical point of glutamic acid). After cooling over night, a white crystalline solid was isolated by filtration. The amino acid was purified once more, by recrystallisation from water and the yield was 70%.

CONCLUSIONS

The enzymatic synthesis of ^{15}N labelled L-glutamic acid starting from ammonium salt has several advantages over the chemical synthesis methods, mainly a good yield in use of $^{15}NH_4Cl$, accompanied by the formation of naturally occurring isomer. The results of this experiment show an 99% at. ^{15}N enrichment of L-glutamic acid and no isotope dilution. This method can be easily adapted for large scale preparation purpose by immobilization of GDH on a solid matrix.

Both experimental and theoretical study on the NMR spectra of L-glutamic acid were carried out. The determination of ^{15}N enrichment of amino acid was realized by ^{13}C NMR spectroscopy and suppose quantitative analysis of ^{13}C in the region 48-58 ppm. Our result is in agreement with that obtained using a conventional method, gas chromatographic-mass spectroscopy (GC/MS). The optimized geometry was performed by using HF as well as B3LYP density functional method with the 6-31G(d), 6-311G(d,p) and 6-311+G(d,p) basis sets. The best results were obtained by DFT/B3LYP/6-311+G(d,p) method. Accordingly, the DFT/B3LYP method was addopted for the following NMR computation. The ^{13}C simulated chemical shifts are in good agreement with the experimetal data (exception for carboxyl carbon nuclei in the neutral zwitterionic form) but for coupling constants determination the problem of basis set remains open to further investigation.

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