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Professional paper

SYNTHESIS AND SPECTROSCOPIC INVESTIGATIONS OF *β-L*-ASPARTYL-CYCLOHEXYLAMIDE AS POTENT LIGAND FOR TRANSITION METAL COMPLEXES

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Abstract

L-aspartic acid is a natural amino acid and a building block for peptides and proteins. Besides, a series of compounds and metallic complexes such as metalloproteins which contain acid residues have biological applications. In this course, a new route of preparation of *β-L*-aspartyl-cyclohexylamide was carried out by regioselective acylation of cyclohexylamine using *N*-phthaloyl-*L*-aspartic anhydride, followed by hydrolysis of phthaloyl group with hydrazine hydrate.
The preparation of *β-L*-aspartylamide was attempted by an original method which involved 4 steps of synthesis. In the first synthetic step, *N*-carbethoxyphthalimide was used through a method developed in our group, managing to improve the conditions of reaction to not affect the chiral center. The second activation phase was performed in good yields, obtaining the pure product. In the third stage, the amine regioselectively attacked the *β* position and in the last step, the amide deprotection was achieved by hydrazinolysis. The structure of the intermediates and the final amide product was confirmed by HRMS, ^{1}H- and ^{13}C-NMR.

Keywords: L-aspartic acid, synthesis, aspartyl-amides, spectral analysis.

1. Introduction

L-aspartic acid (1, Scheme 1) is one of the most important amino acids with an additional carboxylic group in the side chain (Bregier-Jarzebowska, Gasowska, Jastrzab, & Lomozik, 2009). The degree of nerve tissue degradation brought on by Alzheimer's disease can be determined by comparing the concentration of free L-amino acids, such as aspartic and glutamic acid, in the brain tissues with their comparison to the condition to those of healthy individuals (Perry, Berry, Hansen, Diamond, & Mok, 1971., Perry, Hansen, Berry, Mok, & Lesk, 1971., Saifer, 1971., Tarbit, Perry, Perry, Blessed, & Tomlinson, 1980., Smith, Bowen, Francis, Snowden, Neary, 1985., Fisher, Lorenzo, Abe, Fujita, Frey, Emory, D'Aniello, 1998., D'Aniello, Fisher, Migliaccio, Cammisa, D'Aniello, & Spinelli, 2005). In the central nervous system, this acid is an important neurotransmitter (Vaccarino, Schwartz, Hartigan, & Leckman, 1995., Curtis, & Crawford, 1969., Krnjevic, 1970., Aprison, Shank, Davidoff & Werman, 1968., Bennett, & Balcar, 1999., Chen, Gabelle, Stansfeld, Johnston, Yuan, Jacob, Snyder, Traynelis & Wyllie, 2005). It participates in the thermogenic processes induced by prostaglandin E1 (PGE1) and it is a component of the active centers of certain enzymes (Monda, Viggiano, Sullo, & De Luca, 1998). According to some reports, aspartic acid functions as a bridging ligand, and appears as a bidentate or tridentate ligand (Kryger & Rasmussen, 1973., Antolini, Menabue, Pellacani, & Marcotrigiano, 1982., Yasui & Ama, 1975., Bukietynska, Podsiadly, & Karwecka, 2003., Nomiya & Yokoyama, 2002., Legler, Kazachenko, Kazbanov, Per'yanova, & Veselova, 2001., Komiyama, Igarashi, & Yukawa, 2008). This is of course as a consequence of the three possible donor sites, two carboxylic and one amine group (Bregier-Jarzebowska, Gasowska, & Lomozik, 2008., Lehninger, Nelson & Cox, 2005). By comparing the complexes formed with a variety of metal ions of the same valency at pertinent pH ranges, it is

possible to study its coordination behavior (Bukietynska, et al., 2003., Legler, et al., 2001., Komiyama, et al., 2008).

L-aspartic acid is a naturally occurring amino acid widely used for the synthesis of peptides, α and β aspartylamides and other synthetic derivates with significant biological functions. Given the biochemical importance of L-aspartic acid, its derivates and metal complexes, this study proposes a new strategy for the synthesis of β-L-cyclohexylaspartyl amide using a four-step method.

To synthesize the predesigned final product, it was necessary in the very beginning to protect the α-amino group. N-phthaloylation has long been recognized as a method for protecting of primary amino groups, particularly during peptide synthesis (Boissonnas, 1963). Because of the known complications associated with the use of phthalic anhydride in amino acid reactions, the method of Nefkens (Nefkens, 1960., Nefkens, Tesser, & Nivard, 1960) using N-carbethoxyphthalimide (2) was chosen in the protection stage.

2. Experimental Section

Materials and instrumentation

All reagents and chemicals were purchased from commercial sources and used as received. TLC monitoring was performed by using silica gel 60 F_{254} on aluminum sheets (Merck®), and visualization was achieved under UV at λ=254 nm; I_2 bath or ninhydrin spray). NMR spectra were recorded on Bruker® AM 600 instruments operating at 600 and 150 MHz for ^{1}H and ^{13}C nuclei, respectively. All chemical shifts (δ value) are given in ppm without TMS added. The chemical shifts were measured against the solvent residual peak. Melting points were measured on an ELECTROTHERMAL® instrument and were not corrected. Mass spectra were carried out on an LTQ ORBITRAP® XL (Thermo Scientific) instrument which was externally calibrated using the manufacturer's APCI or ESI(+) calibration mix. The samples were introduced into the spectrometer by direct infusion.

***N-phthaloyl-L-aspartic acid* (3)**. 6 g (0.0566 mol) Na_2CO_3 were dissolved in 30 mL H_2O and then 3 g (0.0225 mol) *L*-aspartic acid (**1**), was added at room temperature. The mixture was cooled at 0 to -8 °C and then 5.4 g (0.0082 mol) of *N*-carbethoxyphthalimide (**2**) were added. The mixture was stirred until practically all the *N*-carbethoxyphthalimide had gone into solution (ca. 30 min). After standing in this temperature range for 24 h, the solution was filtered to remove ethyl carbamate and acidified with 6N HC1 (10.3 mL) to pH = 1. An oil separated, which crystallized upon cooling or scratching. After being allowed to stand overnight in the fridge, crystals were collected, washed with mother solution and dried. For purification, the product For purification, the product (**3**) was suspended in 22.5 mL of 1M Na_2CO_3 solution until pH = 11.5, and reprecipitated with 7 mL of 6N HCl until pH = 1. $C_{12}H_9NO_6$ (**3**), White solid; MW = 263.203; Yield 42%, mp = 178-180 °C; (Lit.(Homsi & Kasideh, 2015): 193 °C); TLC analysis yielded a single spot (R_f = 0.5) (ethanol:acetone = 3:1 (*v*/*v*) (visualization under UV at λ = 245 nm); MS (ESI+, CH_3OH) $[M+Na]^+$: 286.0315, Exact Mass: 263.043; ^{1}H-NMR (DMSO-d_6, 600MHz, δ/ppm): 7.87-7.93 (overlapped signals, 4H from aromatic rest), 5.15 (triplet, 1H, H_2), 2.88-2.96 and 3.12-3.18 (two doublets, 2H, H_3). ^{13}C-NMR (DMSO-d_6, 150MHz, δ/ppm): 171.94; 170.35; 167.50; 135.39; 131.59; 123.91; 48.39; 34.32.

***N-phthaloyl-L-aspartic anhydride* (4).** Into a 50 mL round-bottomed flask with a stirring bar were placed 5.459 g (0.0207 mol) *N*-phthaloyl-*L*-aspartic acid (**3**) and 27 mL (0.2841 mol) acetic anhydride. The mixture was refluxed for 3 h at 90-100 °C under nitrogen atmosphere. An opalescent solution with white precipitate were formed. The precipitate was isolated by filtration, washed with cold ether and dried in a desiccator over NaOH or KOH for 4-5 days to give 2.237 g (44%) of compound (**4**). $C_{12}H_7NO_5$ (**4**), White solid; MW = 245.1877; Yield 44%, mp = 223-225 °C; TLC analysis yielded a single spot (R_f = 0.46) (ethanol:acetone = 3:1

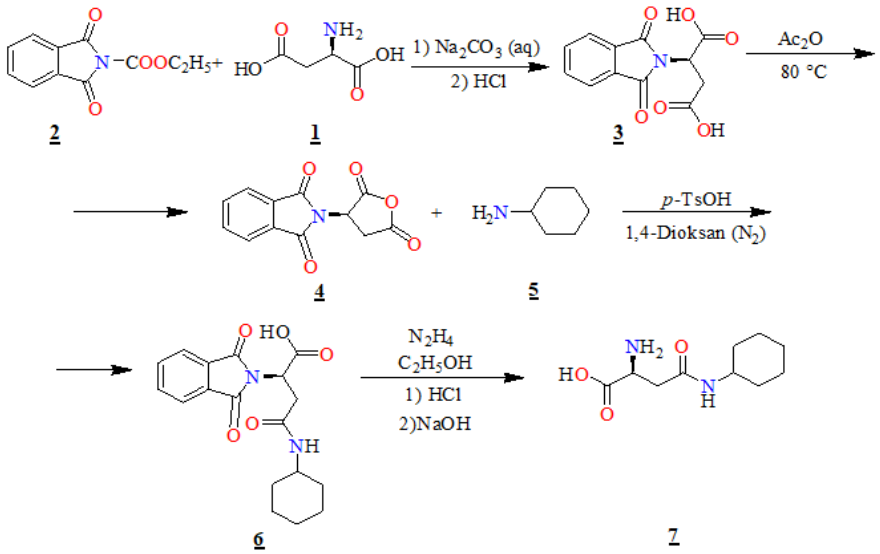
(*v*/*v*) (visualization under UV at λ = 245 nm); ^{1}H-NMR (DMSO-d_6, 600MHz, δ/ppm): 7.9-7.98 (overlapped signals, 4H from aromatic rest), 5.60 (triplet, 1H, H_2), 3.35-3.40 and 3.45-3.50 (two doublets, 2H, H_3). ^{13}C-NMR (DMSO-d_6, 150MHz, δ/ppm): 170.48; 169.85; 167.01; 135.63; 131.54; 124.26; 47.73; 33.84.

***N-phthaloyl-β-L-cyclohexylaspartylamide* (6).** Into a 50 mL round-bottomed flask were placed 0.852 g (0.00347 mol) *N*-phthaloyl-*L*-aspartic anhydride (**4**), 0.45 mL cyclohexylamine (**5**) in 10 mL 1,4-dioxane and 20 mg crystals of *p*-toluenesulfonic acid as catalyst. A mixture was refluxed for 15 h at 60 °C, afterwards solvent was evaporated, the solid was filtered and recrystallized from ether. $C_{18}H_{20}N_2O_5$ (**6**) Beige solid; MW = 344.372. The product was extremely unstable and it was not possible to make measurements for its identification. After the isolation it was directly used for the next synthesis.

***β-L-cyclohexylaspartylamide* (7)**. Into a 100 mL round-bottomed flask, 0.914 g (0.002654 mol) *N*-phthaloyl-*β*-*L*-cyclohexylaspartylamide (**6**) in 30 mL ethanol was treated with 0.58 mL (0.011943 mol) hydrazine hydrate 100%. Solution was refluxed for 2 hours at 80 °C, cooled at room temperature and allowed to stand for overnight at fridge. The precipitate was filtered, treated with 10 mL HCl 0.5N and stirred for 30 min at room temperature. Phthalic hydrazide was removed by filtration and the solution was treated with 1M Na_2CO_3 until pH = 5 and stayed for overnight at fridge. The crystals obtained were filtered, washed with ether and dried in a vacuum desiccator. $C_{10}H_{18}N_2O_3$ (**7**), White solid; MW = 214.2615; Yield 31%, mp = 220-223 °C (desc.); MS (ESI+, CH_3OH) $[M+H]^+$: 215.1386, Exact Mass: 214.2615; ^{1}H-NMR (CD_3COOD, 600MHz, δ/ppm): 1.24-1.36 (multiplet, 5H axial from cyclohexyl rest); 1.60-2.06 (overlapped signals, 5H equatorial from cyclohexyl rest); 4.33-3.69 (1H, H_2; 2H, H_3; 1H, N; 2H, N), 11.46 (singlet, 1H, –COOH group); ^{13}C-NMR (CD_3COOD, 150MHz, δ/ppm): C_1 (177.15); C_4 (170.32); C_2 (51.57); C_5 (48.93); C_3, C_6, C_7, C_8, C_9, C_{10} (34.40; 32.09; 32.04; 31.83; 25.15; 24.52).

3. Results

The ligand *β*-*L*-Cyclohexylaspartylamide (**7**), namely (*S*)-2-amino-4-(Cyclohexylamino)-4-oxobutanoic acid, was obtained through a method analogous to the peptide synthesis, under mild conditions (*Scheme 1*). The method involves four steps of synthesis. The reactions of the first, second and third step were monitored by TLC in ethanol/acetone = 3/1.



Scheme 1. Synthesis of *β-L*-cyclohexylaspartylamide (**7**) by phthaloylation method

In the first step, the identity of the compound (**3**) was confirmed by spectrometric analysis (HRMS), and spectroscopic means (^{1}H-NMR and ^{13}C-NMR spectra, *Figure 1a-c*).

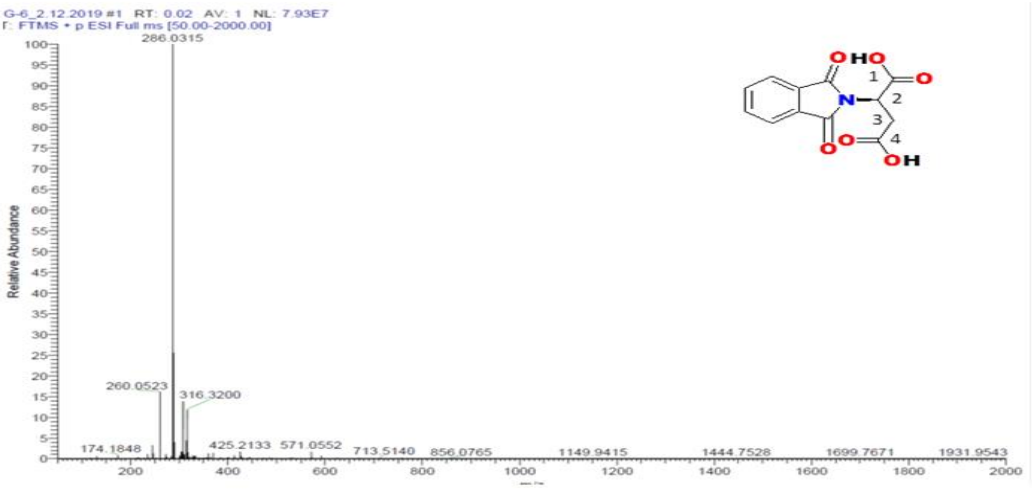


Figure 1a. HRMS spectrum (ESI) of *N*-phthaloyl-*L*-aspartic acid (**3**)

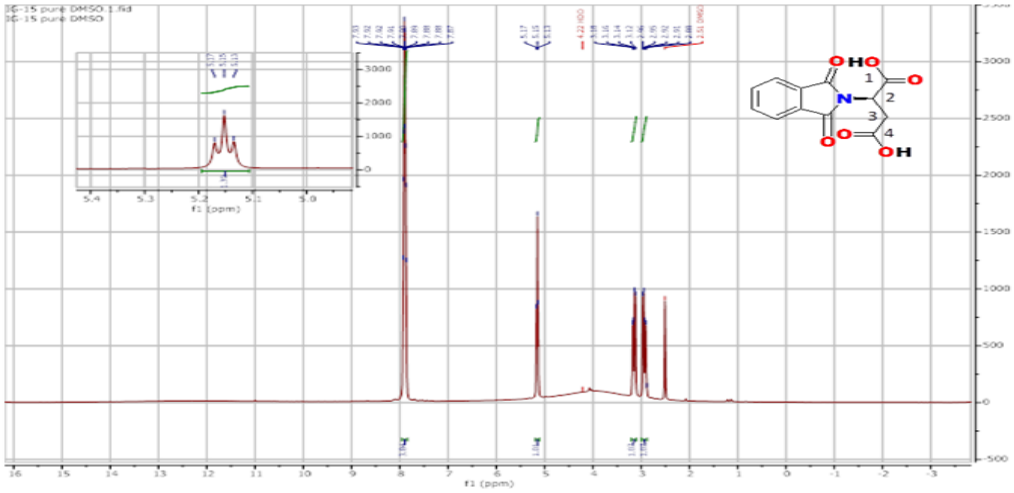


Figure 1b. ^{1}H-NMR spectrum of *N*-phthaloyl-*L*-aspartic acid (**3**)

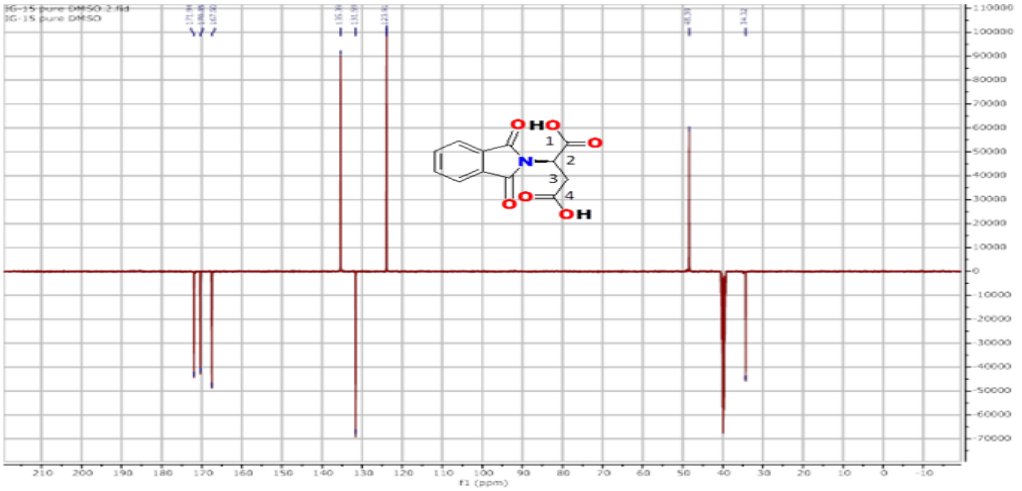


Figure 1c. ^{13}C-NMR spectrum of *N*-phthaloyl-*L*-aspartic acid (**3**)

After the successful protection of the amine group, protection of both carboxylic groups was undertaken, by reaction with the acetic anhydride, obtaining the second product, *N*-phthaloyl-*L*-aspartic anhydride (**4**). This product was also confirmed by ^{1}H-NMR and ^{13}C-NMR spectra. They are given only in the tabular form in the experimental.[1] Afterwards, the third synthetic step was developed, by nucleophilic attack of the amine at the *β* carbonyl group. The amine that was successfully employed was cyclohexyl amine, but the product was very unstable so it was directly used in the last synthetic step. In the last step, the identity of the compound *β-L*-cyclohexylaspartylamide (**7**), was confirmed by spectrometric analysis (HRMS), and spectroscopic means (^{1}H-NMR and ^{13}C-NMR spectra, *Figure 2a-c*).

[1] The spectra of the second product are not given in the caption Results due to the space limitations! On the other hand, the values are given tabular in the Experimental and discussed in details in the Discussions!

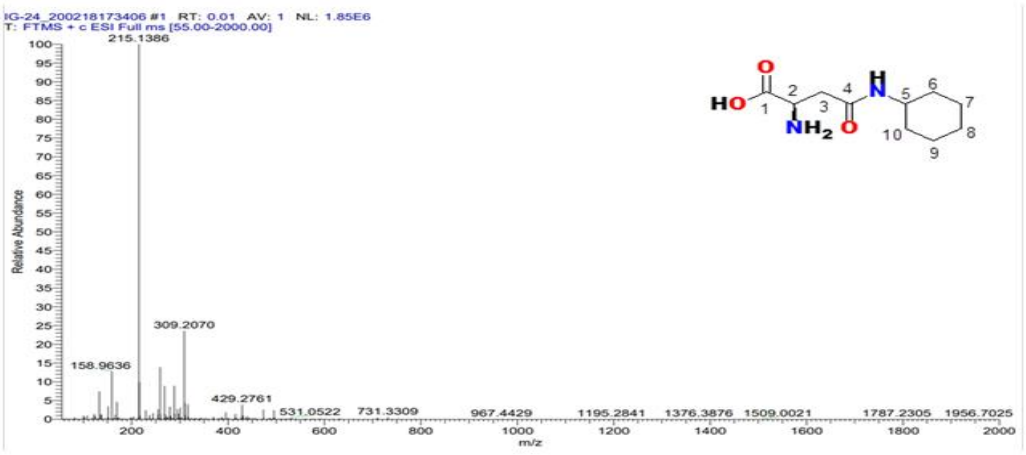


Figure 2a. HRMS spectrum (ESI) of *β-L*-cyclohexyl aspartyl amide (7)

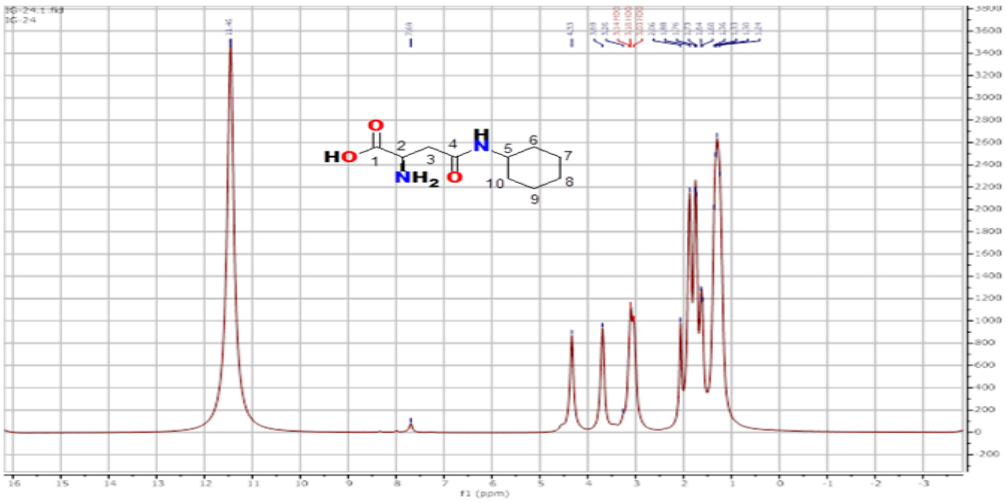


Figure 2b. ^{1}H-NMR spectrum (ESI) of *β-L*-cyclohexylaspartylamide (7)

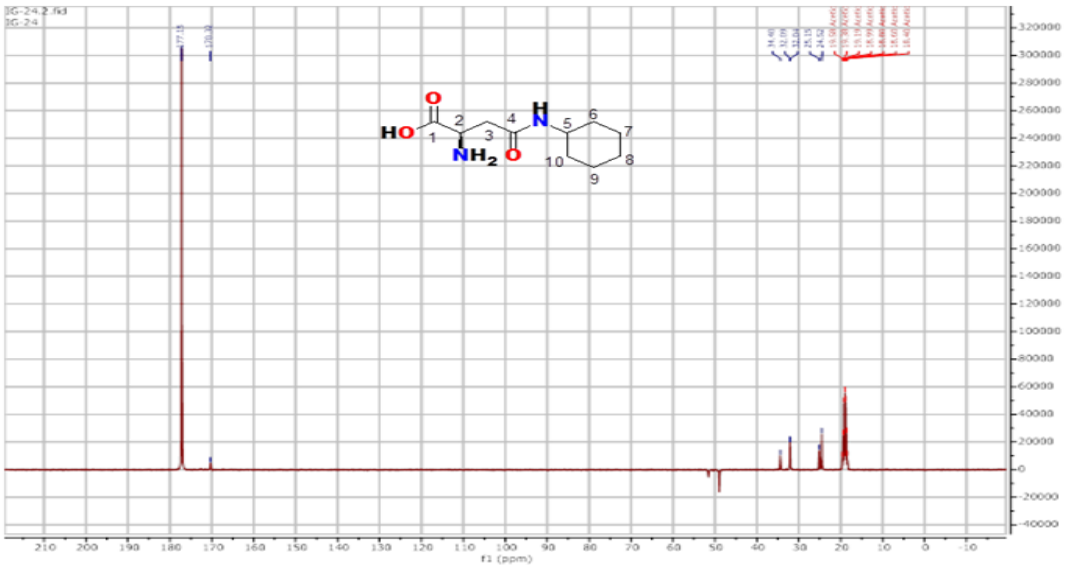


Figure 2c. ^{13}C-NMR spectrum (ESI) of *β-L*-cyclohexyl aspartyl amide (**7**)

4. Discussion and Conclusions

This favorable method for the preparation of amides consists of using the phthaloyl group as an amino-protecting group in the first step. Some authors employed acylating groups like phthalic anhydride (Homsi, et al., 2015., Piutti, 1885., Piutti, 1886) and phthalic acid (Homsi, et al., 2015) in the first step of the synthesis. Nefkens in 1960 had suggested *N*-carbethoxyphthalimide (**2**) as a reagent that gives good yields and can be easily used in the protection phase for blocking the amino group (Nefkens, 1960., Nefkens, et al., 1960). The same procedure, the methodology of direct introducing the phthaloyl group with the *N*-carbethoxyphthalimide (**2**) has been tried with other amino acids, while it has not been tried with an aspartic acid (**1**). Thereby, the reaction between *L*-aspartic acid (**1**) and *N*-carbethoxyphthalimide (**2**) was developed for the first time, in alkaline aqueous solution at low temperature (-8 - 0 °C), overnight. After the workup, the product (**3**) as white crystalline compound was isolated. The identity of the compound (**3**) was confirmed by spectrometric analysis (HRMS), and spectroscopic means (^{1}H-NMR and ^{13}C-NMR spectra, *Figure 1a-c*).

The expected product (**3**) has a calculated molecular mass of 263.043. A peak with the highest intensity (100%) was observed at a value of 286.0315 *m/z* corresponding to the $[M+Na]^+$ ion cluster in the high-resolution mass spectrum (*Figure 1a*), indicating high agreement with the expected product mass (**3**).

The NMR spectra for the compound (**3**) were recorded in DMSO-d_6, at 600 MHz. The ^{1}H-NMR spectrum of *N*-protected amino acid (**3**) showed peaks in the region 7.87-7.93 ppm with the integral of 4, characteristic for the four protons of the aromatic ring, and at 5.15 ppm a triplet signal was observed (zoomed part in *figure 1b*), as expected for the single proton at the chiral center (marked with position 2 at the proposed structure). Furthermore, two doublet signals were noticed, one on 2.88-2.96 and the other on 3.12-3.18 ppm, each by integral 1. Those doublets are regarded as two protons at position 3, and as different doublets because the resolution is very high, so it can be distinguished that they are slightly different due to the chirality of the proton next to them, at position 2.

The ^{13}C-NMR spectra (*Figure 1c*) was recorded in the DEPT mode. On the spectrum were noticed 8 signals. The aromatic part has 6 carbon atoms, but because of the symmetry there are three (equivalent) pairs showing two positive signals (doublets) on 135.39 and 123.91, and one negative signal (singlet) at 131.59 ppm; two carbons from the heterocyclic part are equivalent and give one negative signal at 167.50, and the chiral carbon

shows a positive signal on 48.39 ppm. Finally, the signals at 171.94, 170.35 and 34.32 ppm were assigned to three different carbons from the amino acid part. The first two belong to the carbonyl carbons and the third one to the aliphatic carbon at position 3. Peaks that appears in the ^{1}H-NMR, ^{13}C-NMR spectra, together with the MS confirmed the proposed structure of the product (**3**).

The second step predicts activation of the carboxylic group in the β position, which is carried out simultaneously with the protection of the carboxylic group at the α position through the formation of an intramolecular anhydride. To realize this, *N*-protected amino acid (**3**) and acetic anhydride were refluxed under nitrogen atmosphere, producing *N*-phthaloyl-*L*-aspartic anhydride (**4**).

The NMR spectra for the compound (**4**) were also recorded in DMSO-d_6, at 600 MHz. The ^{1}H-NMR spectrum of *N*-phthaloyl-*L*-aspartic anhydride (**4**) displayed peaks in the region 7.9-7.98 ppm with integral of 4, characteristic for the four protons of the aromatic ring, and at 5.6 ppm a triplet signal was observed as expected for the single proton at the chiral center. Also, two doublet signals were noticed, one on 3.35-3.40 with integral 1.4 and the other on 3.45-3.50 ppm with integral 0.84, this corresponds to a full 1:1 integral. Those doublets are regarded as two aliphatic protons at position 3. In this case, not only because of the high resolution but also because the system is rigid due to the cyclization, which makes them different and as a consequence two separated signals appeared in the spectrum.

The ^{13}C-NMR spectra were recorded in the DEPT mode. On the spectrum, 8 signals were noticed. The aromatic part has six carbon atoms, but due to symmetry, there are three (equivalent) pairs showing two positive signals (doublets) at 135.63 and 124.26, and one negative signal (singlet) at 131.54 ppm. Additionally, two carbons from the heterocyclic part are equivalent and give one negative signal at 167.01, and the chiral carbon shows a positive signal at 47.73 ppm. Finally, the two negative signals at 170.48, and 169.85 belong to the carbonyl carbons from the amino acid part, and one positive signal at 33.84 ppm to the aliphatic carbon at position 3.

The third synthetic step consists of a regioselective condensation in the *b* position of the *N*-phthaloyl-*L*-aspartic anhydride with aromatic and aliphatic amines not mentioned in the literature. Initially, it was tried with piperazine, but no satisfactory results were obtained. Subsequently, cyclohexylamine (**5**) was used, under an inert atmosphere, in 1,4-dioxane and *p*-toluenesulfonic acid as catalysts, which led to the expected results. Unfortunately, product (**6**) showed to be very unstable so it was used immediately in the next synthetic step. This was the last step, thus the amide deprotection was achieved by hydrazinolysis using hydrazine hydrate 100% in ethanol under reflux. At the pH = 5 free amides (**7**) as a white solid was obtained.

The chemical structure and purity of the ligand (**7**) were confirmed by HRMS (ESI), ^{1}H-NMR and ^{13}C-NMR (*Figure 2a-c*). The HRMS (ESI+) spectrum (*Figure 2a*) of *β-L*-cyclohexylaspartylamide (**7**) confirmed the presence of $[M+H]^{+}$ peak, as a molecular peak at 215.1386 *m/z*.

The NMR spectra of the compound (**7**) were recorded in acetic acid-d_4, at 600 MHz. The ^{1}H-NMR spectrum of ligand (**7**) was shown in Fig. 2b. The multisignals within 1.24-1.36 ppm range were assigned to five axial protons of the cyclohexyl rest. The overlapped signals within 1.60-2.06 ppm were assigned to the five equatorial protons belonging to the cyclohexyl rest. These multisignals include the single proton from H_5. The CH_2, CH, NH and NH_2 groups are also included in these multiplets and in two signals at 3.69 and 4.33 ppm. The signal at 11.46 was assigned to the proton of -COOH group.

The ^{13}C-NMR spectra were recorded in the DEPT mode. The spectrum of ligand (**7**) showed 10 signals. In the proposed structure for ligand (**7**) there are six methylene groups (CH_2) and in ^{13}C-NMR spectrum (*Figure 2c*) six positive signals were recorded in the region from 24.52 to 34.40 ppm, which is very characteristic for aliphatic groups. The two carbonyl carbons also give positive signals at high shifts at 170.32 and 177.15, which is characteristic for the carbons of the carbonyl groups. The negative signal at 48.93 was assigned to C_2 and the negative signal at 51.57 was assigned to C_5.

In this study, the main goal was to synthesize *β-L*-aspartylamides via a mild phthaloylation. The amide bond formation process was applied for this purpose, from peptide synthesis starting with an *N*-terminal amino acid.

In the protection stage, *N*-carbethoxyphthalimide (**2**) as used as an acylating agent to introduce a phthaloyl group, a procedure that was developed for the first time, in an alkaline aqueous solution at low temperature. Afterwards, the amine that was successfully employed was cyclohexyl amine. *β-L*-cyclohexylaspartylamide (**7**) has not been alluded in the literature. The compound's identity was confirmed by spectral analysis and this one can be used as a potent ligand to form novel complexes with transition metals.

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