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Synthesis and characterization of a new peptide analogue: 2-Amino-N-(2-amino-ethyl)-3-phenyl-propionamide

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ABSTRACT

In the present study, a new dipeptide analogue namely 2-amino-amino ethyl)-3-phenyl-propionamide was synthesized by liquid phase synthesis from L-phenylalanine and ethylenediamine. The intermediates and the final compound were analyzed by analytical thin layer chromatography and characterized by ^1H NMR, D_2O exchange NMR and mass spectral analyses to ascertain the purity and elucidate the chemical structure. This is the first experimental report of the synthesis and characterization of this compound.

Key words: Boc, phenylalanine, ethylenediamine, peptides.

INTRODUCTION

Nuclear medicine is a branch of medicine and medical imaging that uses the nuclear properties of matter in diagnosis and therapy. More specifically, nuclear medicine is a part of molecular imaging because it produces images that reflect biological processes that take place at the cellular and sub-cellular level. Nuclear medicine procedures use pharmaceuticals that have been labeled with radionuclides (radiopharmaceuticals). In diagnosis, radioactive substances are administered to patients and the radiation emitted is detected. The diagnostic tests involve the formation of an image using a gamma camera or positron emission tomography. Imaging may also be referred to as radionuclide imaging or nuclear scintigraphy. Other diagnostic tests use probes to acquire measurements from parts of the body, or counters for the measurement of samples taken from the patient In nuclear medicine, nearly 95% of the radiopharmaceuticals are used for diagnostic purposes while the rest are used for therapeutic purpose. Radiopharmaceuticals usually have no pharmacological effects because in most cases where they are used for diagnostic purposes [1, 2].

Recently, a number radiolabelled peptides (bioactive or inactive) and peptide analogues have proven to be useful diagnostic imaging agents. Due to their small size, these molecules exhibit favorable pharmacokinetic characteristics, such as rapid uptake by target tissue and rapid blood clearance, which potentially allows images to be acquired earlier following the administration of a labelled peptide radiopharmaceutical [3]. Peptides are important regulators of growth and cellular functions not only in normal tissue but also in tumors. So they are becoming radioligands of increasing interest in nuclear oncology for targeted tumor diagnosis and therapy. Therefore, development of new peptide radiopharmaceuticals is becoming one of the most important areas in nuclear medicine research. In the present study, we attempted synthesis of a new dipeptide analogue namely 2-amino-N-(2-amino-ethyl)-3-phenyl-propionamide and its chemical characterization by different spectral means.

MATERIALS AND METHODS

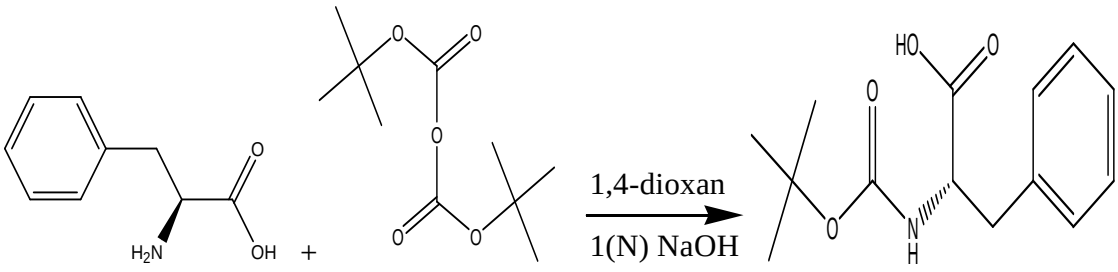
Reagents and chemicals: Di-tertiary-butyl-dicarbonate or Boc anhydride ($C_{10}H_8O_5$), sodium carbonate and silica gel (for column chromatography) from Sisco Research Lab. Pvt. Ltd.; L-phenylalanine, ethylenediamine, 1,4- dioxan, 1-hydroxy benzotriazole, N,N'-dicycloamino pyridine, sodium hydroxide, sodium chloride, sodium sulphate, diphosphorous pentoxide, ethyl acetate, dimethyl formamide, ninhydrin and silica gel G (for thin layer chromatography) from Merck; potassium bisulphate, amberlyst-15 resin, sephadex LH-20 from Sigma Aldrich. All the other reagents and chemicals were of highest purity grade obtained commercially. Doubled-distilled water from all-glass-still was employed throughout the studies.

Ninhydrin solution: Ninhydrin is Indanerioninhydrate ($C_6H_4COCOCO.H_2O$). Ninhydrin solution used to develop colour in TLC plates was 1:1 mixture of solution A and solution B, where 50 ml solution A was composed of 100 mg ninhydrin in 50 ml acetone and 50 ml solution B was composed of 3 ml acetic acid, 1.5 ml pyridine and 45.5 ml acetone.

Brine solution: Brine is supersaturated sodium chloride solution used to remove water from organic mixtures. Supersaturated sodium chloride (brine) solution was prepared by dissolving sodium chloride in hot water until sodium chloride deposits.

Synthesis protocol: The details of synthesis are described below [4,5].

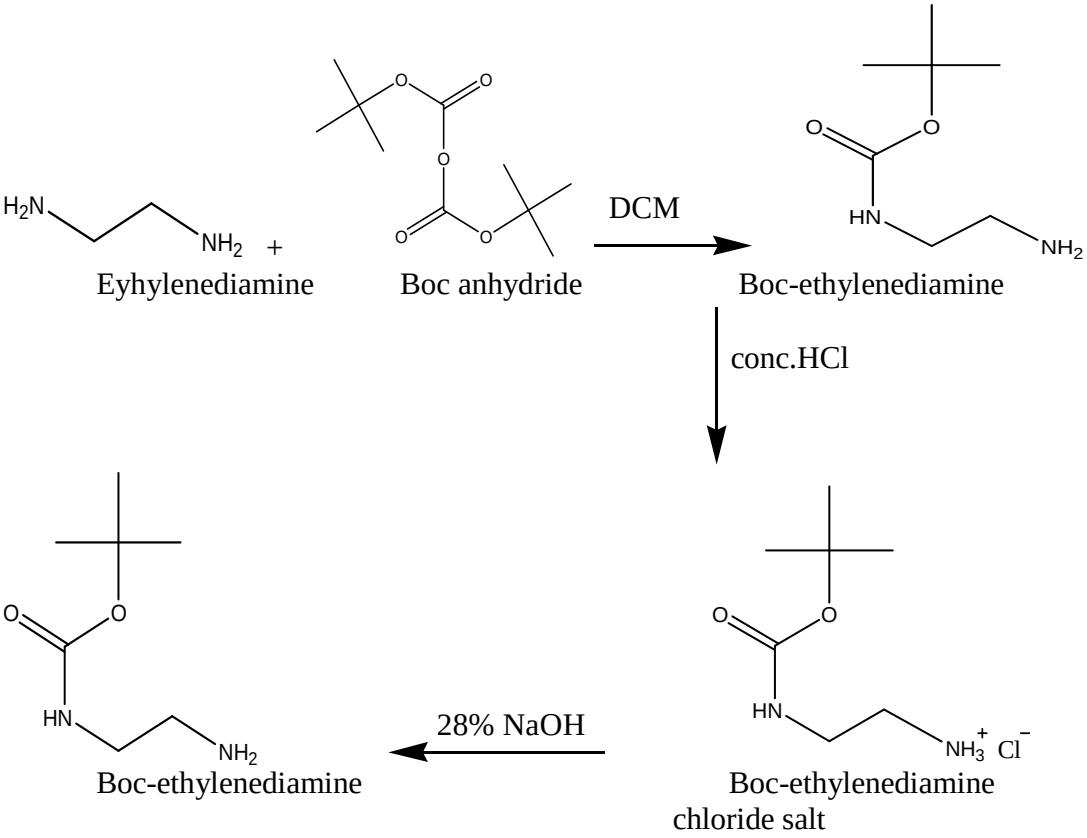
1. N-Boc protection of L-phenylalanine
Chemical reaction:



L-phenylalanine Boc anhydride Boc-phenylalanine

Scheme 1: Chemical synthesis of Boc-phenylalanine.

2. N-Boc protection of ethylenediamine
Chemical reaction:



Scheme 2: Chemical synthesis of Boc-ethylenediamine.

Synthetic procedure: 2.5 g phenylalanine and 22 ml 1, 4-dioxan was taken in 100 ml round bottom flask and the mixture was vigorously stirred for nearly about 15 to 20 minutes. Then 13 ml 1(N) sodium hydroxide (NaOH) solution was added to this solution. The mixture was again vigorously stirred for another 20 minutes keeping in ice-bath. Then 6 ml Boc anhydride was added to this solution, drop by drop, using dropping funnel. Additional amount of 1, 4-dioxan (minute amount) was added for washing the container containing Boc. The reaction mixture was stirred overnight at room temperature. The reaction mixture was made acidic (pH 2-3) drop by drop, with 2(N) potassium bisulphate ($KHSO_4$) solution. Acidity of the solution was checked with pH paper. The reaction mixture was then concentrated in rotary evaporator.

Work up procedure: The concentrated reaction mixture was extracted 3 times with ethyl acetate in 1:1 ratio by volume in a separating funnel. The ethyl acetate extract was again passed through brine solution and then heated sodium sulphate was added to absorb any moisture present in the extract. Finally the product was concentrated at rotary evaporator and kept in a vacuum desiccator with phosphorous pentoxide (P_2O_5). The final product obtained was a highly viscous liquid with golden yellow appearance. The synthesized product was analyzed on a TLC plate and compared with phenylalanine using 5% methanol in chloroform as mobile phase.

Purification of the product: The synthesized product was purified by silica gel (60-120 mesh) column chromatographic technique using 2% methanol in chloroform as mobile phase.

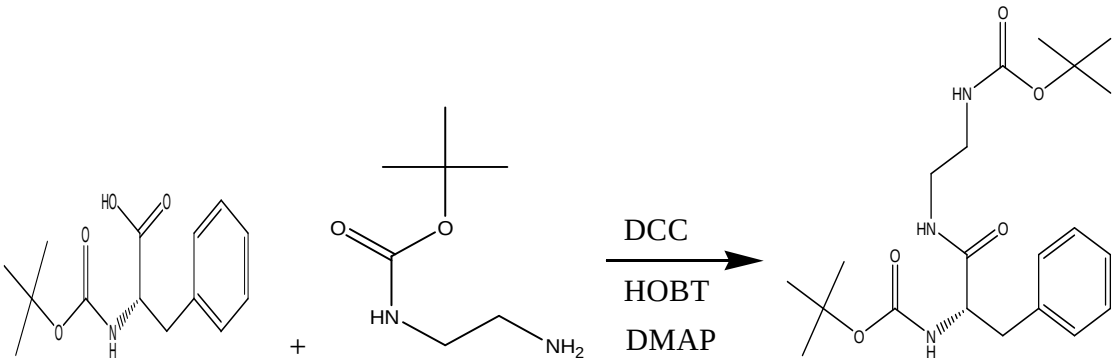
Synthetic procedure: 6.75 g (≡ 7.55 ml) of ethylenediamine was dissolved in 32 ml dichloromethane (DCM) and 3.3 g (≡3.5 ml) of Boc anhydride was dissolved in 3 ml of DCM. Then the above two solutions were mixed under vigorous stirring condition for 1 hr using a magnetic stirrer. Reaction mixture was then stirred for another 1 hr while cooling at 20^0C.

Work up procedure: 38 ml water was slowly added to the reaction mixture over a time period of 45 minutes. Mixture was stirred for 15 minutes followed by phase separation and aqueous phase (i.e. lower layer) was removed. 20 ml water was added and pH was adjusted to 1.5 with concentrated HCl. Reaction mixture was stirred for 15 minutes followed by phase separation and removal of the organic phase (upper layer). 32 ml DCM was added and pH was adjusted to 12 with 28% NaOH solution. Reaction mixture was again stirred for 15 minutes. Finally the organic phase containing the product was collected and the aqueous phase was extracted several times with DCM for higher yield. The collective DCM extract was washed with brine and then treated with heated Na_2SO_4 for absorption of any moisture present in the extract. It was then concentrated at a rotary evaporator and kept in a vacuum desiccator with P_2O_5. The synthesized product was analyzed on a TLC plate and compared with ethylenediamine using 10% methanol in chloroform as mobile phase.

Purification of the product: The synthesized product was purified by silica gel (60-120 mesh) column chromatographic technique using 5% methanol in chloroform as mobile phase.

3. Coupling between N-Boc protected L-Phenylalanine and N-Boc protected Ethylenediamine

Chemical reaction:



Scheme 3: Chemical synthesis of Boc protected coupled product.

Synthetic procedure: In a 250 ml 2 necked round bottom flask 3.65 gm of Boc-phenylalanine was dissolved in min volume of dry DCM. Then 2.42 gm of Boc-ethylenediamine was added to it followed by addition of 2.05 gm of HOBT (1-hydroxybenztriazole) and 1.85 g of DMAP (4-dimethyleaminopyridine). Then the environment of reaction was made nitrogenous and cooled by ice-salt mixture for 10-15 minutes. 3.12 g of DCC (N, N'- dicyclohexyl carbodiimide) was taken in a conical flask and dissolved in minimum volume of DCM. This DCC solution was

injected to the reaction mixture in cold condition. Finally few drops of DMF were added to the solution and then the reaction mixture was stirred over night. After the reaction the reaction mixture was analyzed on a TLC plate using 10% methanol in chloroform as mobile phase.

Work up procedure: After the completion of reaction, the reaction mixture was filtered to remove DCU (Dicyclohexyl urea). The filter was concentrated at rotary evaporator. Then it was dried in a vacuum desiccator (using P_2O_5) over night. The next day the dried product was dissolved in methyleacetate and filtered to remove DCU. Then it was taken in a separating funnel and then solution was washed with concentrated 1(N) HCl. HCl was added slowly by repeated addition of very small volume until the upper layer was acidic. Then 1(N) Na_2CO_3 solution was added drop by drop to make the solution acid free. Finally the excess acid was removed by washing with brine solution. The filtrate was taken in a conical flask and heated Na_2SO_4 was added and kept for about 1 hr. Then the solution was filtered, taken in a weighed round bottom flask and concentrated at rotary evaporator. Finally it was kept in vacuum desiccators with P_2O_5. The synthesized product was analyzed on a TLC plate and compared with phenylalanine and ethylenediamine using 5% methanol in chloroform as mobile phase.

Purification of the product: The synthesized product was purified by silica gel (60-120 mesh) column chromatographic technique using 2% methanol in chloroform as mobile phase.

Crystallization of the purified product: The product purified by column chromatography was dried in vacuum desiccators over night. At first the dry product was dissolved in chloroform and heated to evaporate most of the chloroform. Then it was taken on hot water and ice cold hexane was added drop by drop. The mixture was readily cooled on cold water. Crystals of the product appeared as gelatinous mass. It was filtered on a Buckner funnel and the filtrate was dried by desiccation.

Cleaning of resin: 10 gm Amberlyst-15 resin was taken and soaked in 50 ml methanol for 24 hour. After soaking with methanol it was washed with 4(M) ammonia prepared in methanol. It was neutralized by washing with fresh methanol (indicated by no colour change of pH paper). Then the neutralized resin was stirred for another 1 hour with 3(M) HCl prepared in 50% methanol. Again it was neutralized by washing with fresh methanol until no colour change occurs in pH paper.

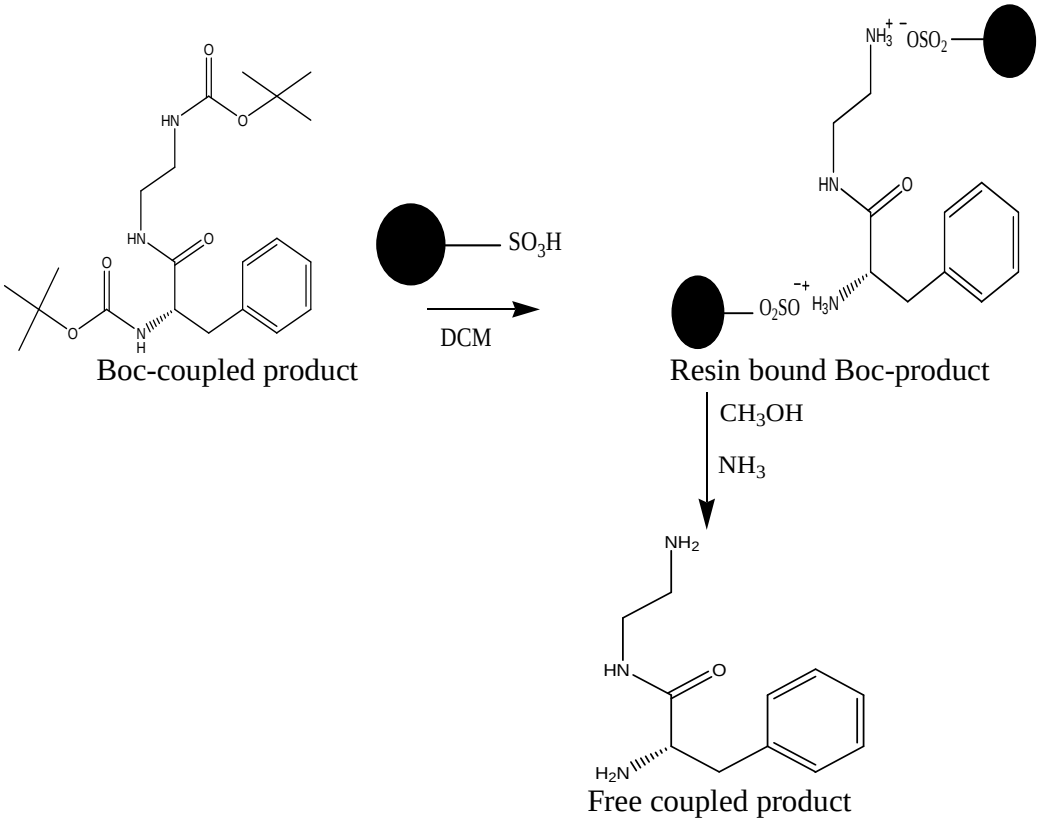
Work up procedure: 600 mg of dried coupled product was taken and dissolved in 20 ml of DCM. 4 g of cleaned Amberlyst-15 resin was taken and added to the product dissolve in DCM. The mixture was stirred over night on magnetic stirrer at room temperature. After 16 hours of stirring the reaction mixture was analyzed on a TLC plate and compared with the Boc protected coupled product using 5% methanol in chloroform as mobile phase. No spot from the reaction mixture developed on the TLC plate.

After 20 hours of stirring the reaction mixture was again analyzed on a TLC plate and compared with the Boc protected coupled product using 5% methanol in chloroform as mobile phase. Now presence of free coupled product was seen. The reaction mixture was filtered through Hertz funnel. Residue on the funnel was at first washed with DCM and then with methanol. The filtrate was collected in a conical flask. The resin part was then stirred for 1½ hour with 4(M) NH_3 in

methanol. The reaction mixture was spotted on a precoated TLC plate against the coupled product using 3% methanol in chloroform as mobile phase. The reaction mixture was filtered through Hertz funnel followed by methanol wash. It was collected in a previously weighed 50 ml round bottom flask and concentrated at rotary evaporator and was then kept in a vacuum desiccator using P_2O_5 over night. The next day an oily product appeared on the bottom of the round bottom flask. It was analyzed on a TLC plate using 3% methanol in chloroform as mobile phase.

4. Removal of BOC from the coupled product 2-Amino-N-(2-amino-ethyl)-3-phenyl-propionamide

Chemical reaction:

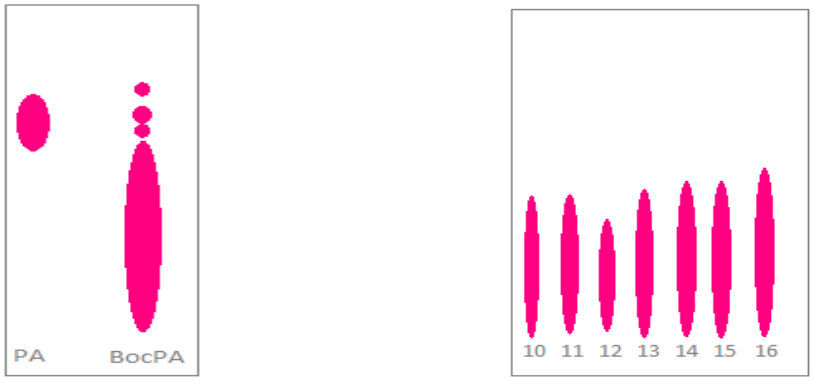


Scheme 4: Recovery of the free coupled product.

Purification of the final product: The product was at first dissolved in double distilled water by adding few drops of methanol in it and nonpolar impurities were then extracted with chloroform in a centrifuge for 2-3 minute. After that the aqueous part was concentrated and purified by sephadex LH-20 gel chromatography. The pure fractions were collected in a test tube and lyophilized to obtain the final product.

RESULTS

N-Boc protection of L-Phenylalanine
Expected theoretical yield = 15.51 g.
Practical yield of Boc-phenylalanine = 8.52 g.
% of yield of Boc-phenylalanine = 54.93%
Purification:



(a) (b)

Fig. 1: (a) Readily synthesized Boc-phenylalanine analyzed on TLC and compared with Phenylalanine (b) Purified fractions of Boc-phenylalanine analyzed on TLC.

Characterization:

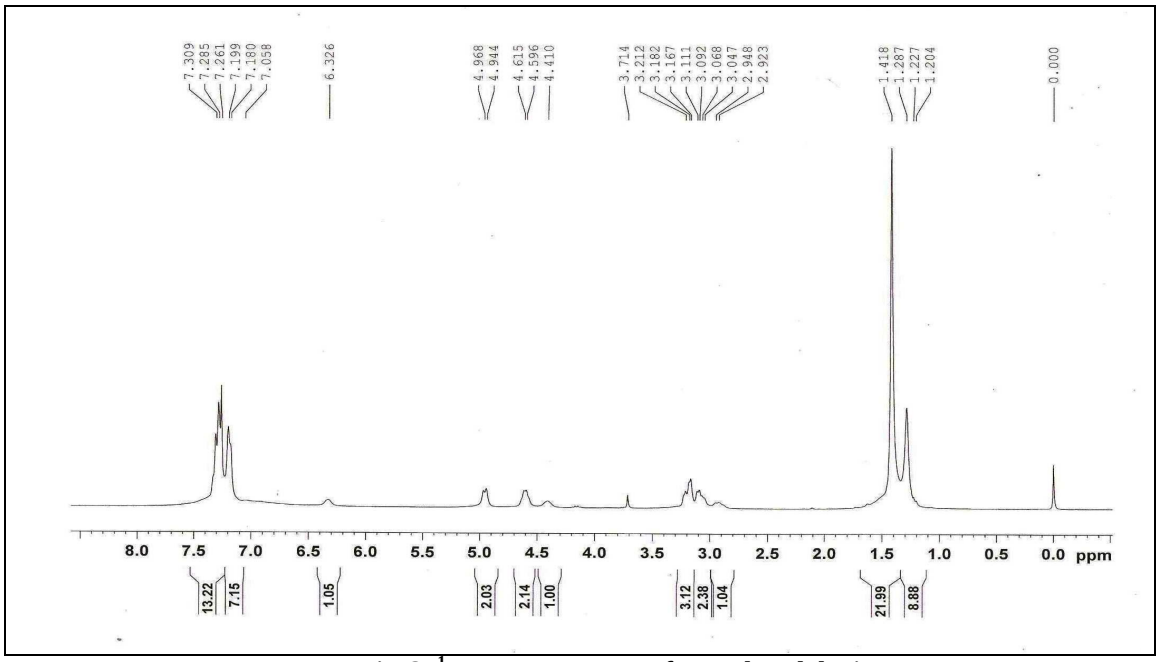


Fig. 2: ^{1}H NMR spectrum of Boc-phenylalanine.

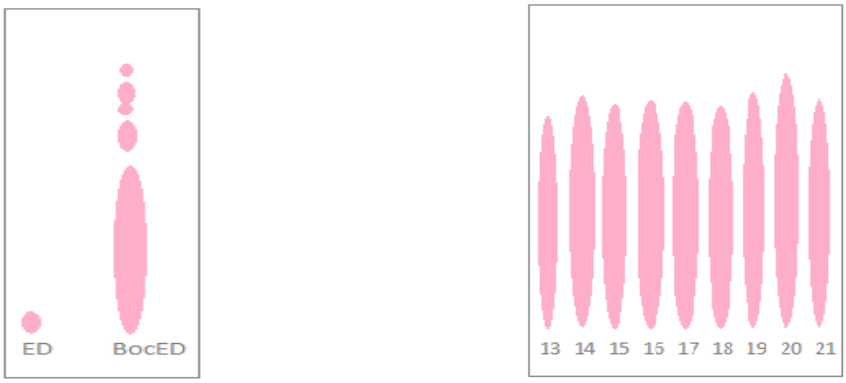
^{1}H NMR values:

Node	Shift	Split	Comment (ppm relative to TMS)
CH_3	1.204-1.287	triplet	tertiary butyl
CH_2	3.047-3.111	multiplet	methylene
CH	4.41	singlet	methane
NH	6.326	singlet	secondary amide

CH 7.058-7.199 triplet aromatic

2. N-BOC protection of Ethylenediamine

Expected theoretical yield = 18 g.
Practical yield of Boc-ethylenediamine = 3.09 g.
% of yield of Boc-ethylenediamine = 17.17%
Purification:



(a) (b)

Fig. 3: (a) Readily synthesized Boc-ethylenediamine analyzed on TLC and compared with Ethylenediamine. (b) Purified fractions of Boc-ethylenediamine analyzed on TLC.

Characterization:

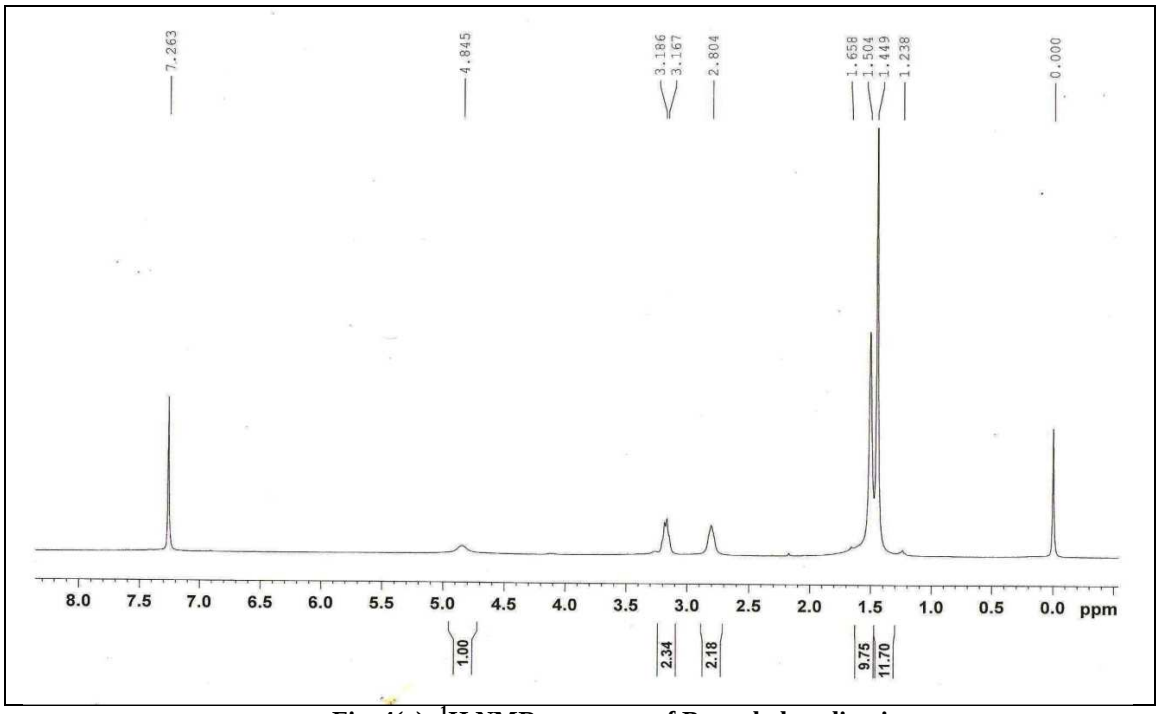


Fig. 4(a): ^{1}H NMR spectrum of Boc-ethylenediamine.

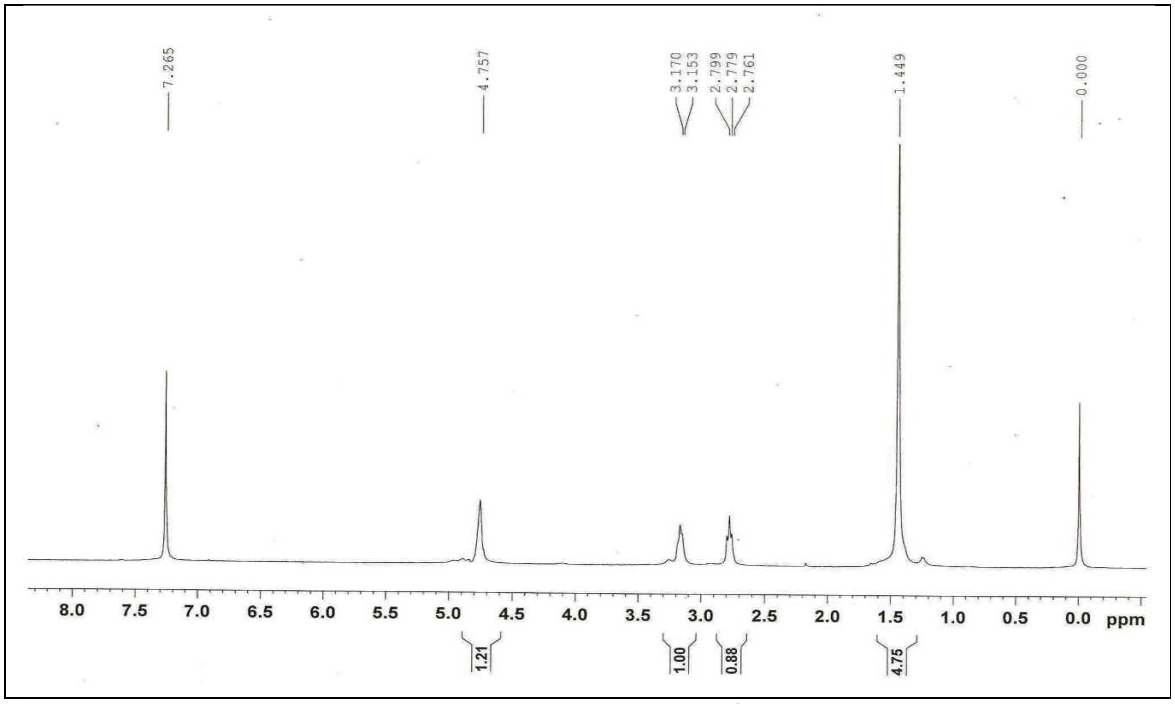


Fig. 4(b): D_2O exchange NMR spectrum of Boc-ethylenediamine.

^{1}H NMR values:

Node	Shift	Split	Comment (ppm relative to TMS)
CH_3	1.449	singlet	tertiary butyl
NH_2	1.504	singlet	primary amine
CH_2	2.804	singlet	methylene (alpha to primary amine)
CH_2	3.17	doublet	methylene (beta to primary amine)
NH	7.263	singlet	secondary amide

The absence of the singal at 1.504(δ value) and the persistence of the singal at 1.449(δ value) in D_2O exchange NMR determines the presence of an primary amine group and a tertiary butyl group in the compound.

3. *Coupling between Boc-phenylalanine and Boc-ethylenediamine*

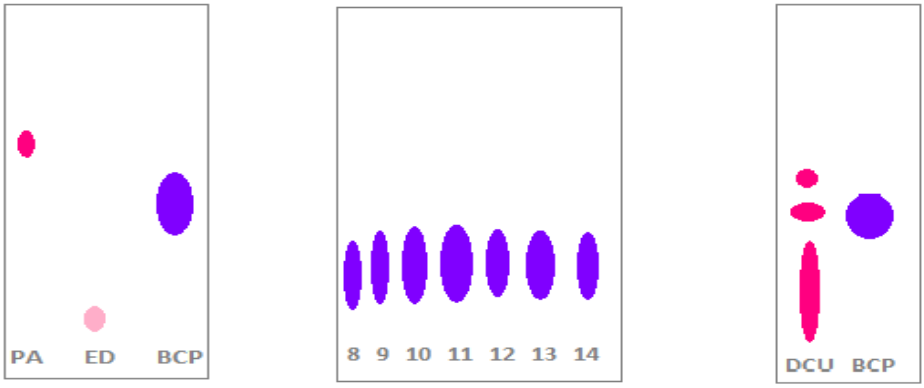
Yield:

Expected theoretical yield = 5.68 g.

Practical yield of Boc protected coupled product = 2.08 g.

% of yield of Boc protected coupled product = 36.62%

Purification:



(a) (b) (c)

Fig. 5: (a) Readily synthesized Boc protected coupled product analyzed on TLC and compared with Phenylalanine and Ethylenediamine. (b) Purified fractions of Boc protected coupled product analyzed on TLC. (c) Purified Boc protected coupled product analyzed on TLC and compared with Dicyclohexylurea.

Characterization:

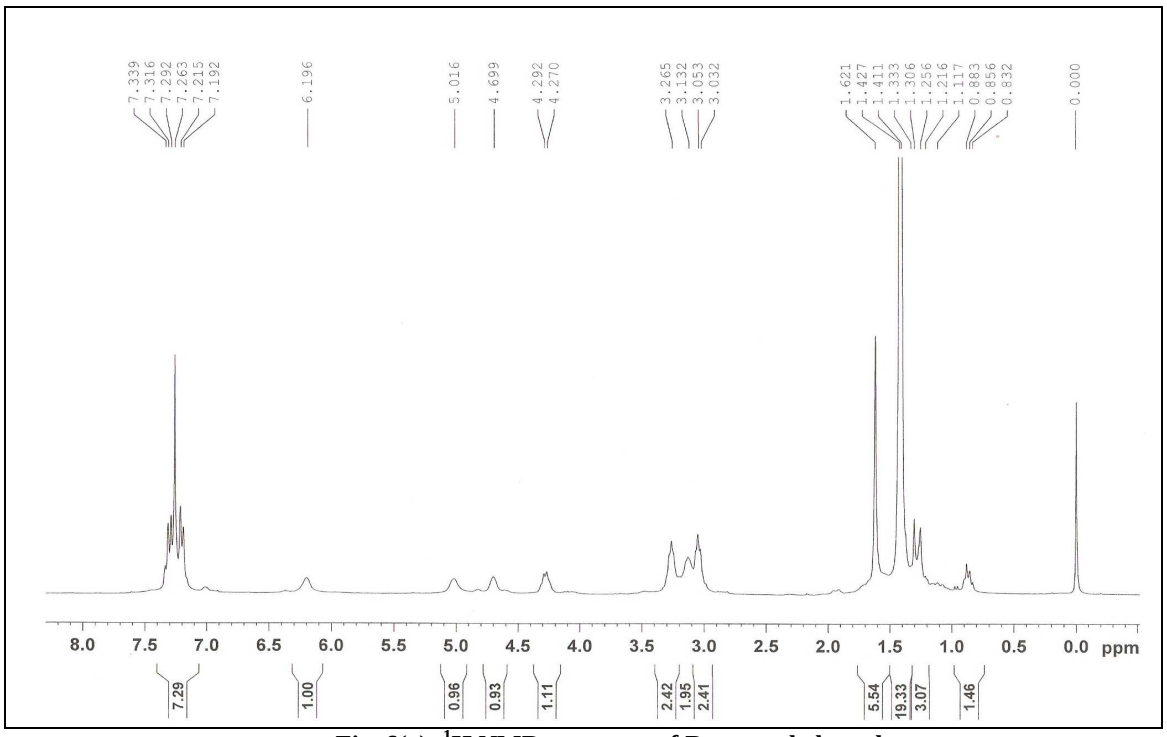


Fig. 6(a): ^{1}H NMR spectrum of Boc-coupled product

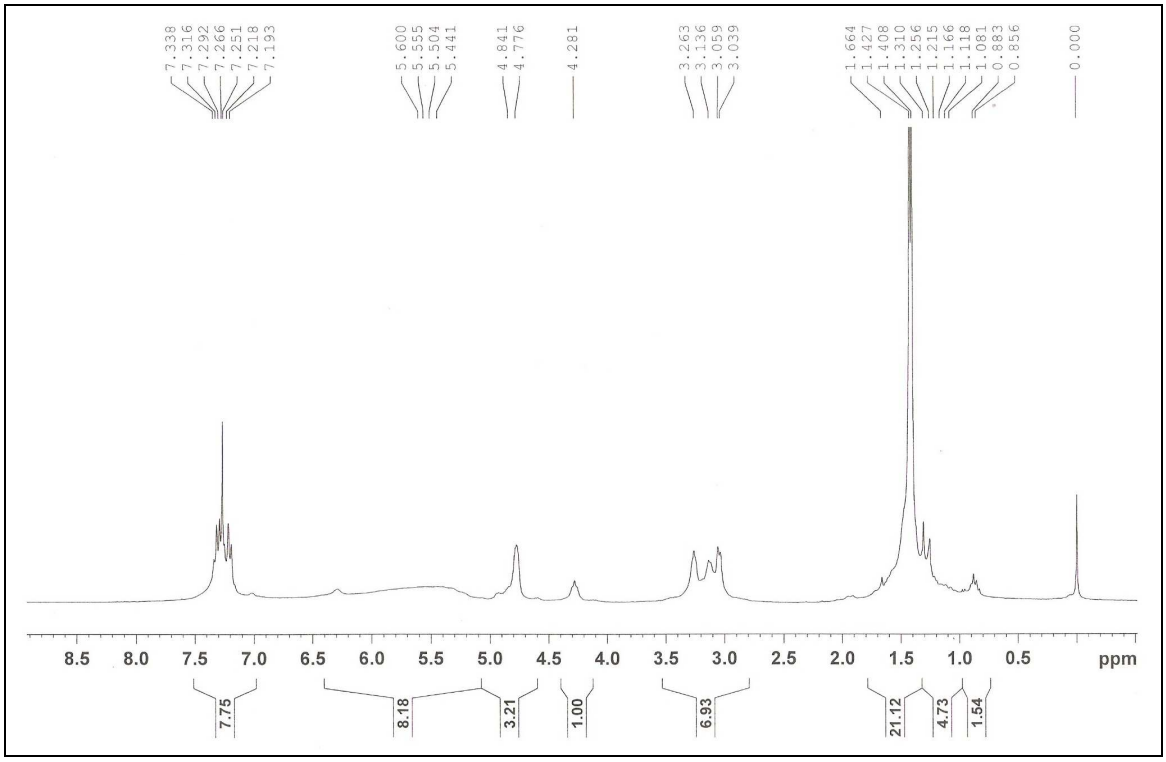


Fig. 6(b): D_2O exchange NMR spectrum of Boc-coupled product.

^1H NMR values:

Node	Shift	Split	Comment (ppm relative to TMS)
CH_3	1.621	singlet	tertiary butyl
CH_2	3.04	doublet	methylene (alpha to phenyl)
CH_2	3.132	singlet	methylene (beta to butyloxycarbonyl)
CH_2	3.265	singlet	methylene (gamma to butyloxycarbonyl)
CH	4.28	doublet	methane
NH	6.196	singlet	secondary amide
CH_2	7.192-7.339	multiplet	phenyl

The persistence of the signal at 1.621(δ value) in D_2O exchange NMR determines the presence of a tertiary butyl group in the compound.

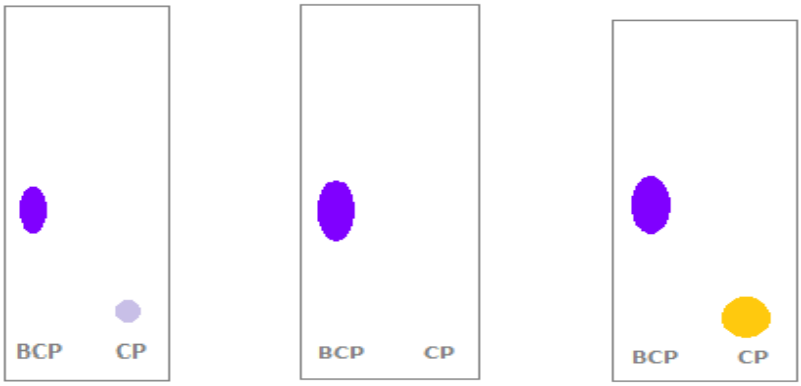
4. *Boc removal of coupled product*

Expected theoretical yield = 0.31 g

Practical yield of unprotected coupled product = 0.22 g

% of yield of unprotected coupled product = 70.97%

Purification:



(a) (b) (c)

Fig. 7: (a) Presence of Boc protected coupled product in the reaction mixture at the beginning of the Boc removal reaction. (b) Most of the coupled is bound to the resin after 16hours of stirring. (c) Presence of free coupled product analyzed on TLC and compared with Boc protected coupled product.

Fig. 8: Fractions of coupled product purified by sephadex LH 20 gel chromatography.

Characterization:

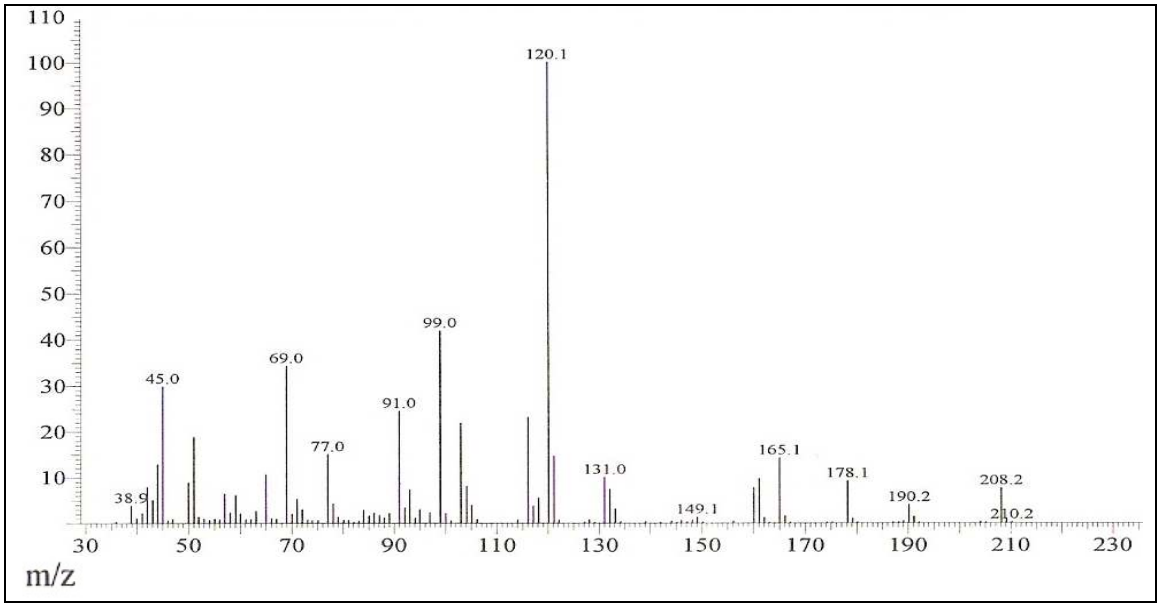


Fig. 9: Mass spectrum of the coupled product.

The mass spectrum shows the molecular ion (M) peak at m/z value 208.2 and an associated (M+2) peak at m/z 210.2. The base peak shows m/z 120.1 which reflects most abundant fragment $C_6H_5CH_2CH^+NH_2$.

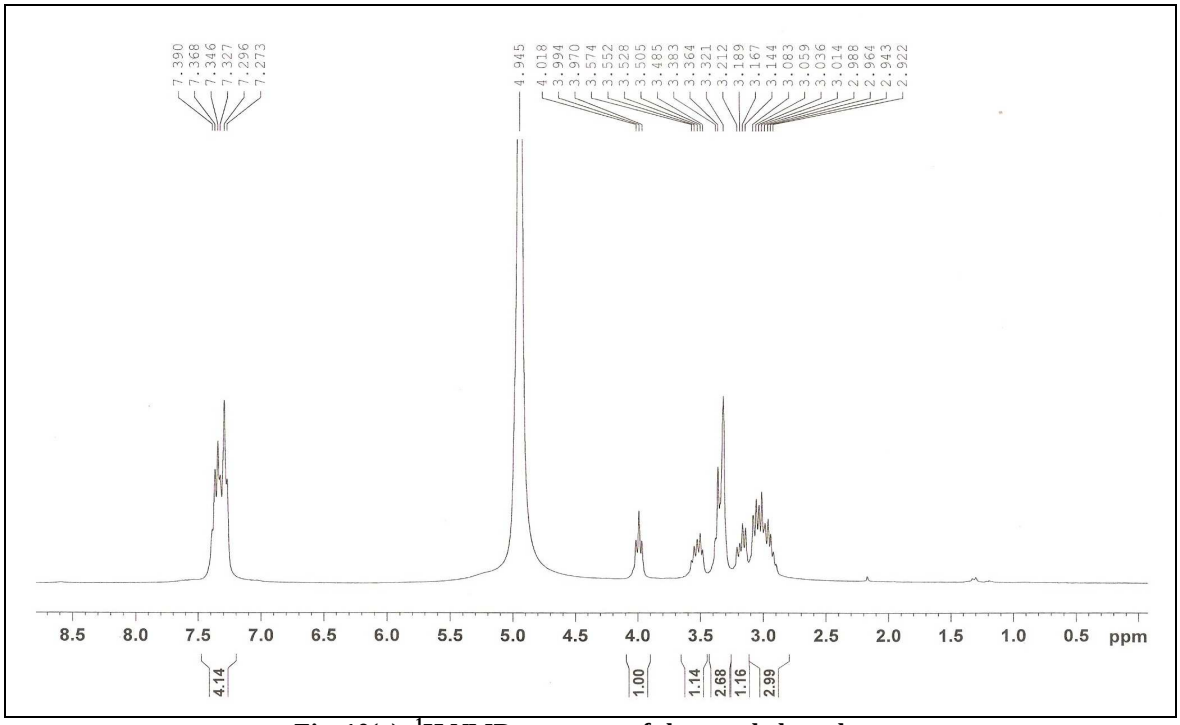


Fig. 10(a): ^{1}H NMR spectrum of the coupled product.

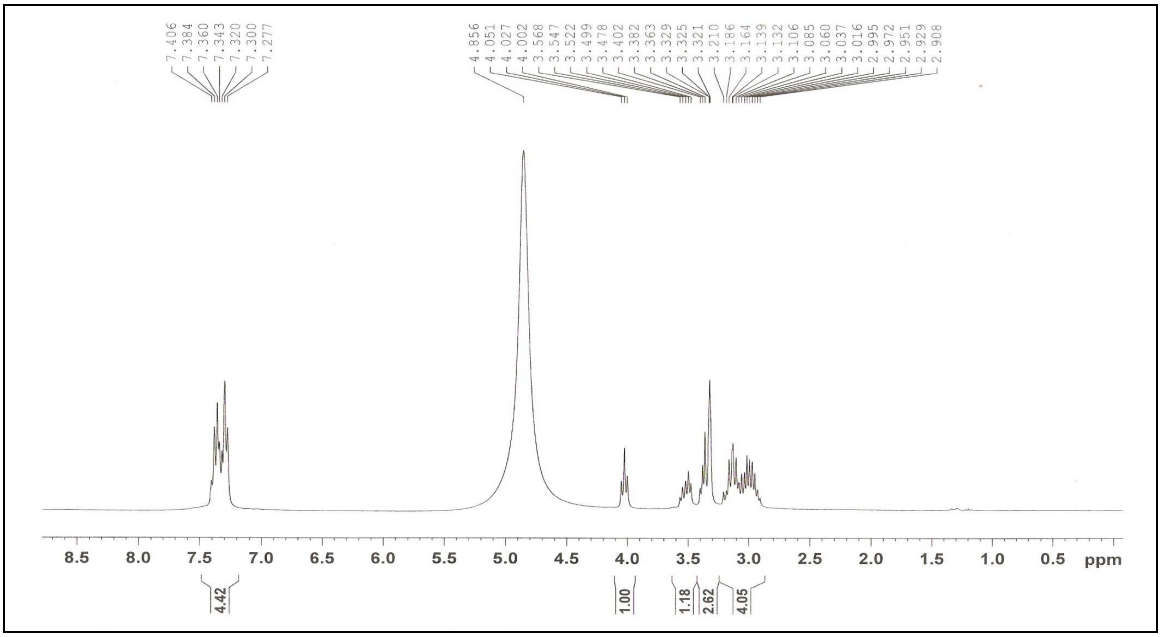


Fig. 10(b): D_2O exchange NMR spectrum of the coupled product.

^{1}H NMR values:

Node	Shift	Split	Comment (ppm relative to TMS)
CH_2	2.92-3.083	multiplet	methylene (alpha to primary amine)
CH_2	3.167-3.212	multiplet	methylene (alpha to phenyl)
CH_2	3.321-3.383	triplet	methylene (beta to primary amine)
CH	3.97-4.018	triplet	methine

The ^{1}H NMR, D_2O exchange NMR and mass spectral analyses determine the final product to be 2-amino-N-(2-amino-ethyl)-3-phenyl-propionamide.

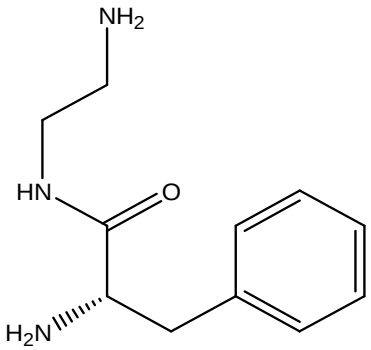


Fig. 11: Final product: 2-amino-N-(2-amino-ethyl)-3-phenyl-propionamide.

DISCUSSION

Nuclear medicine has evolved in recent time as a powerful tool in both medical diagnosis and therapy. The application of radiopharmaceuticals along with gamma camera or positron emission tomography is being extensively used today to diagnose severe pathological conditions like problems associated with peripheral and central blood flow, organ failure and cancer. The diagnostically important radiopharmaceuticals mostly used are peptide or peptide based analogs labeled with nuclides such as ^{99m}Tc, ^{67}Ga, ^{18}F, ^{123}I etc [3].

Amino acids are known to be the building block of proteins but they serve many other functions in cell which are essential for life. It has been established that amino acids also play an important role in the growth of the tumor cells. The involvement amino acid in different physiological process is versatile. This encourages many scientists to radiolabel amino acid based chelating agents with radionucleides and to study their physicochemical and biological behavior. Nature has designed peptides to stimulate, inhibit or regulate many physiological functions. The development of effective peptide-based radiopharmaceuticals for imaging a variety of tumors, infection/inflammation and thrombus has seen a new era in nuclear medicine [6].

In this context, an attempt has been made in the present study to synthesize a small dipeptide analogue namely 2-amino-N-(2-amino-ethyl)-3-phenyl-propionamide by liquid phase synthesis by N-Boc protection of L-phenylalanine and ethylenediamine followed by coupling between N-Boc protected L-phenylalanine and N-Boc protected ethylenediamine and then removal of Boc by resin treatment from the coupled product 2-amino-N-(2-amino-ethyl)-3-phenyl-propionamide i.e. the target dipeptide compound. The intermediates and final compound have been analyzed by analytical thin layer chromatography and characterized by ^{1}H NMR, D_2O exchange NMR and mass spectral analyses the results of which are characteristic to the anticipated chemical structures of the synthesized compounds. The experiments have been performed and repeated successfully with negligible limitations. To the best of our knowledge, this is the first experimental report of the synthesis and characterization of this compound. The encouraging success of this attempt can later be explored for further studies on the synthesized compound including studies on radiolabelling followed by biodistribution *in vivo*, in pursuit of a new radiopharmaceutical.

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