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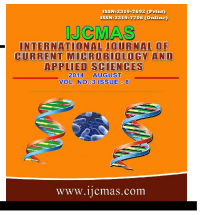

Original Research Article

Synthesis and characterization of Ammonium benzoyltrimethyl chloride

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A B S T R A C T

Interaction of CD40 ligand (CD40L) with CD40 receptor is one of the most immunologically important interactions that involve in the regulation of T cell dependent B cell proliferation, differentiation and antibody production. Therefore, interfering CD40L interaction with CD40 may have important therapeutic applications. Using the three dimensional structure of CD40-CD40L complex, a small CD40L mimetic molecule, Ammonium benzoyltrimethyl chloride (ABTC) was designed by computational techniques. This ABTC was synthesized by benzoylation of trimethylamine and characterized by TLC (Thin Layer Chromatography), chemical tests, Fourier Transform Infrared (FTIR) spectroscopy and Nuclear Magnetic Resonance (NMR) spectroscopy. The FTIR spectrum of ABTC showed the presence of various functional groups such as quaternary nitrogen, carboxyl group, phenyl group and methyl groups. In addition, the ^{1}H NMR spectrum of ABTC confirmed the presence of phenyl group and methyl group hydrognes whereas the ^{13}C NMR spectrum shows the presence of carbonyl group carbon, aromatic carbons and methyl group carbons. As the ABTC mimics CD40L protein, it can be considered as a candidate molecule for the further development of novel immunotherapeutic agent.

Keywords

CD40L, ABTC, FTIR, NMR and mimetic molecule

Introduction

The CD40 receptor is a type I membrane protein that belongs to the nerve growth factor gene family. The CD40 Ligand (CD40L), also known as T-B activating molecule, TNF-related activation protein or gp 39, is a 33 kDa type II membrane glycoprotein expressed on the surface of activated CD4+ T cells [1-6].

CD40 receptor is expressed on several cell types like B cells, Dendritic cells, etc. Binding of CD40L to its receptor CD40 on B cells leads to several effects, including B-cell proliferation, prevention of B cell apoptosis resulting in the establishment of immunological memory, germinal center formation, B cell differentiation,

immunoglobulin production and immunoglobulin class switching [7-11]. Blocking the interaction between CD40 and CD40L leads to several adverse effects like lack of T cell dependent B cell proliferation and antibody production. CD40L mutation is also found in several diseased conditions like hyper IgM syndrome, arthritis, Hodgkin's lymphoma, hypogammaglobulinemia, and viral infections [12-21]. Therefore, providing CD40L could be a strategy in treating these disease conditions. But use of peptides as drug faces problems of drug administration and delivery. In addition, Peptides are unstable and their production is not economical. Use of small molecules as a potent drug has been increasing due to the difficulties in peptide synthesis. Small molecules are found to exert powerful effects on the functions of macromolecules that comprise living systems making it a useful research tool and a pharmacological agent. Small molecules contribute in unprecedented ways to the understanding and betterment of human health [22]. Hence a small molecule that mimics CD40L may prove to be therapeutically effective in treating CD40L deficiency.

Recently, computational methods have been used to discover novel ligands. A collection of small molecules capable of perturbing any disease- related biological pathway is screened using computational methods and this leads to the identification of therapeutic targets [23]. Using these computational methods, many small molecules have been developed for more than 30 targets [24]. These small molecules include the inhibitors of aldose reductase [25], Cyclin dependant kinase 4 (CDK4) [26], matriptase [27], B cell leukemia 2 protein (Bcl- 2) [28], adenovirus protease [29] tyrosine kinase 2 [30] and synthetic agonists of cytokine receptors [31-32].

Using computational methods and the structural information of CD40- CD40L complex, a small mimetic molecule i.e. ammonium benzoyltrimethyl chloride (ABTC) was designed [33]. This small molecule mimics the binding site of CD40L for CD40 receptor. ABTC was synthesized in the laboratory by benzoylation of trimethylamine. This synthesized mimetic molecule was characterized by TLC, chemical tests, FTIR spectroscopy and NMR spectroscopy.

Materials and Methods

Benzoylation of trimethylamine

Trimethylamine (2.8ml, 0.03mol) was taken in a 100ml conical flask and benzoylchloride (3.4ml, 0.03mol) was added with constant shaking and the conical flask was cooled whenever it becomes hot by immersing in cold water. It was vigorously shaken for 5-10 minutes until the odor of benzoyl chloride disappears. The reaction flask was left overnight at room temperature for crystallization of ABTC. Solid crystal like derivative was filtered and purified by recrystallization. The reaction is represented in scheme 1.

Recrystallization of ABTC

4g of ABTC was taken in a 100ml beaker. 30ml of hot ethanol was added into the beaker containing the crude ABTC. It was swirled to dissolve the ABTC. The beaker was placed on the steam bath at 75°C to keep the solution warm. If the solid was still not dissolved, more ethanol was added and swirled again. When the solid was all in solution, it was set on the bench top without disturbing. After 5-7 minutes, ABTC crystals appeared in the flask. Excess of ethanol was filtered out and the recrystallized ABTC was recovered.

Determination of melting point

A few micrograms of ABTC was filled into a capillary tube sealed at one end. The capillary tube was placed in the melting point apparatus (Technico Pvt Ltd., India) and the temperature at which the substance melts was recorded.

Thin layer chromatography

Silica gel was used as an absorbent. Ethanol and Acetic acid in the ratio of 9:1 was used as a solvent. Since ABTC is an organic compound, iodine vapors were used for visualizing the spots. The ABTC and its reactants (trimethylamine and benzoyl chloride) were made to run on the TLC plate. The TLC plate was photographed for record purpose. The Rf values of trimethylamine, benzoyl chloride and ABTC were measured and compared.

Chemical tests

Silver oxide test

1.5g of ABTC was dissolved in 12ml of distilled water in a 50ml conical flask. To this solution, 0.5g of silver oxide was added. This reaction mixture was shaken gently so that the reaction could take place. The formation of effervescence and a precipitate of silver halide were noted. The precipitate was tested for hygroscopic property.

Catalytic test

One gram of bromodiphenyl methane was taken in a 50ml beaker. It was dissolved in 5ml of water. One gram of sodium cyanide was dissolved in 5ml of acetone in another 50ml beaker. When these two solutions are mixed, they are separated into two distinct layers. To the above reaction mixure, 0.5g of the ABTC was added and stirred. Again 0.5g of ABTC was added and stirred well. Reaction or no reaction of clearly separated phases was noted.

Ferric chloride test

To 1% aqueous solution of the ABTC, one drop of 10% ferric chloride was added. The formation of a red colored precipitate was noted.

FTIR Spectroscopy

Dry IR-grade KBr, 200mg was placed in an agate mortar. The KBr was ground to fine powder in the mortar. Two milligrams of ABTC was added to it, which was about 1% of KBr. The ABTC was ground in KBr until it was uniformly distributed throughout the KBr. This mixture was added to the pellet making die to make a pellet. The KBr powder/sample mixture was tamped down on the anvil with the plunger of the die set. A little pressure was applied to the die before it was pulled by vacuum.

The vacuum was applied for 2 minutes at 10,000 pounds of pressure. The vacuum was released first and then the pressure. The base was removed by twisting it off and releasing the 'O' ring seal. The pellet was pushed out using the plunger of the die set with the press to extract the KBr- sample pellet. This sample pellet was subjected to FTIR analysis (Thermo scientific, USA). FTIR spectrum of ABTC was obtained using the in-built Omnic software.

NMR Spectroscopy

The NMR tube was cleaned with HNO_3 to remove metal contaminants. After cleaning with HNO_3, the tube was rinsed with distilled water and then acetone. Finally, the tube was gently warmed for a few minutes for drying.

For preparing the sample for ^{1}H NMR analysis, 5mg of ABTC was dissolved in 0.5ml of the deuterochloroform ($CDCl_3$) whereas for ^{13}C NMR analysis, 20mg of ABTC was dissolved in 0.5ml of CDCl3. To the above sample solution, one drop of tetramethylsilane (TMS) solution was added and it was filtered through a cotton wool straight into the NMR tube to remove any particulate matter.

The NMR tubes containing sample for ^{1}H NMR analysis and ^{13}C NMR analysis were subjected to NMR analysis separately (Brucker, USA). The ^{1}H NMR and ^{13}C NMR spectra of ABTC were obtained using the in-built Topspin 2.0 software.

Results and Discussion

Synthesis of ABTC

Ammonium benzoyltrimethyl chloride (ABTC) was synthesized by benzoylation of trimethylamine. ABTC is a hygroscopic, white, odourless, crystalline powder. The melting point (M.P) of this compound was found to be 288°C. It is soluble in water, ethanol and chloroform. The percentage yield of the ABTC was found to be 22%. It was stored in air tight containers and placed in a desiccator until use.

Purity of ABTC by thin layer chromatography

The ABTC and its reactants, benzoyl chloride and trimethylamine were subjected to separation on silica gel TLC plate. The Rf value of the ABTC was found to be 0.71 which is between the Rf values of benzoylchloride and trimethylamine. The retension factor (Rf) values of reactants, benzoyl Chloride and trimethylamine were found to be 0.8625 and 0.425 (Figure 1). This confirms that ABTC is different from trimethylamine and benzoylchloride. Moreover, the ABTC shows no contaminating trimethylamine and benzoylchloride, suggesting a pure preparation of ABTC.

Chemical tests to determine the quaternary ammonium salt nature of ABTC

Silver oxide test, catalytic test, and the ferric chloride tests were performed for ABTC to confirm that the ABTC is a quaternary ammonium salt.

Silver oxide test

The ABTC with silver oxide produced effervescence and a white precipitate of silver halide, very rapidly. The precipitate was observed to have the property of absorbing water. This test confirmed that ABTC is a quaternary salt.

Catalytic test

The two clearly separated phases of aqueous bromodiphenyl methane and sodium cyanide dissolved in acetone were found to react rapidly when a pinch of the ABTC was added. This test confirmed that ABTC is a quaternary ammonium salt.

Ferric chloride test

The ABTC was found to react with ferric chloride and produced a red colored precipitate of ferric hydroxide. This test also confirmed that ABTC is a quaternary ammonium compound.

FTIR spectroscopy for functional group characterization

As can be seen in Figure 2, the FTIR spectrum of ABTC showed the presence of various functional groups such as quaternary nitrogen at 599.82 cm^{-1}, 578.60 cm^{-1}, 555.46 cm^{-1}, 644.18 cm^{-1} and 618.18 cm^{-1}, carboxyl group at 1718.46 cm^{-1}, phenyl group at 3066.61 cm^{-1} and 3020.32 cm^{-1} and methyl groups at 2854.45 cm^{-1}, 2877.6 cm^{-1} and 2933.53cm^{-1}.

Nuclear Magnetic Resonance Spectroscopy of ABTC for functional group characterization

The ^{1}H NMR and ^{13}C NMR spectroscopic analyses of ABTC were performed to confirm various functional groups in the ABTC. The H^{1} NMR and ^{13}C NMR spectra of ABTC are presented in Figure 3 and Figure 4. The H^{1} NMR spectrum confirmed the presence of its functional groups such as phenyl group at 7.93 ppm, 7.905 ppm, 7.443 ppm, 7.347 ppm and 7.321 ppm and methyl groups at 1.299 ppm, 1.275 ppm and 1.215 ppm.

The ^{13}C NMR spectrum of ABTC confirmed the presence of carbonyl group carbon at 168.692 ppm, aromatic carbons at 128.746 ppm, 130.160 ppm, 130.854 ppm and 133.338 ppm and methyl group carbons at 77.362 ppm, 77.681 ppm and 78.000 ppm.

The CD40L mimetic molecule, ABTC was synthesized by benzoylation of trimethylamine. It is a hygroscopic, white, odourless, crystalline powder. This compound was further characterized by TLC, chemical tests, FTIR spectroscopy and NMR spectroscopy

As ABTC structurally mimics CD40L, it was subjected to binding studies and functional assays such as B- cell proliferation assay and immunoglobulin isotype switching to test the biological activity of this CD40L mimetic molecule [34].

Figure.1 TLC analysis of ABTC: TLC was performed for ABTC. Benzoyl chloride (A) and trimethylamine (B) were also used in the TLC for checking the purity of the ABTC. ABTC shows a descrete band with a Rf of approximately 0.71, suggesting a pure preparation of ABTC

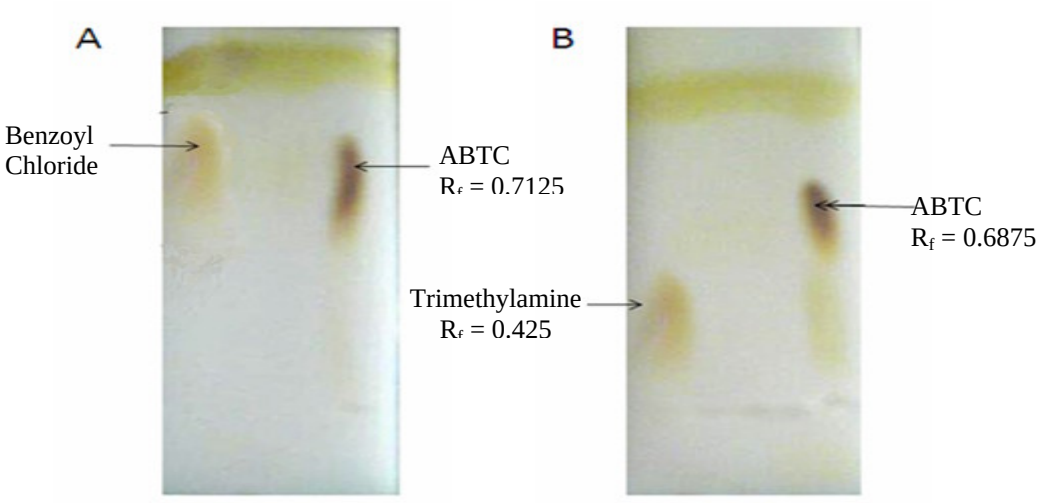


Figure.2 FTIR spectrum of ABTC

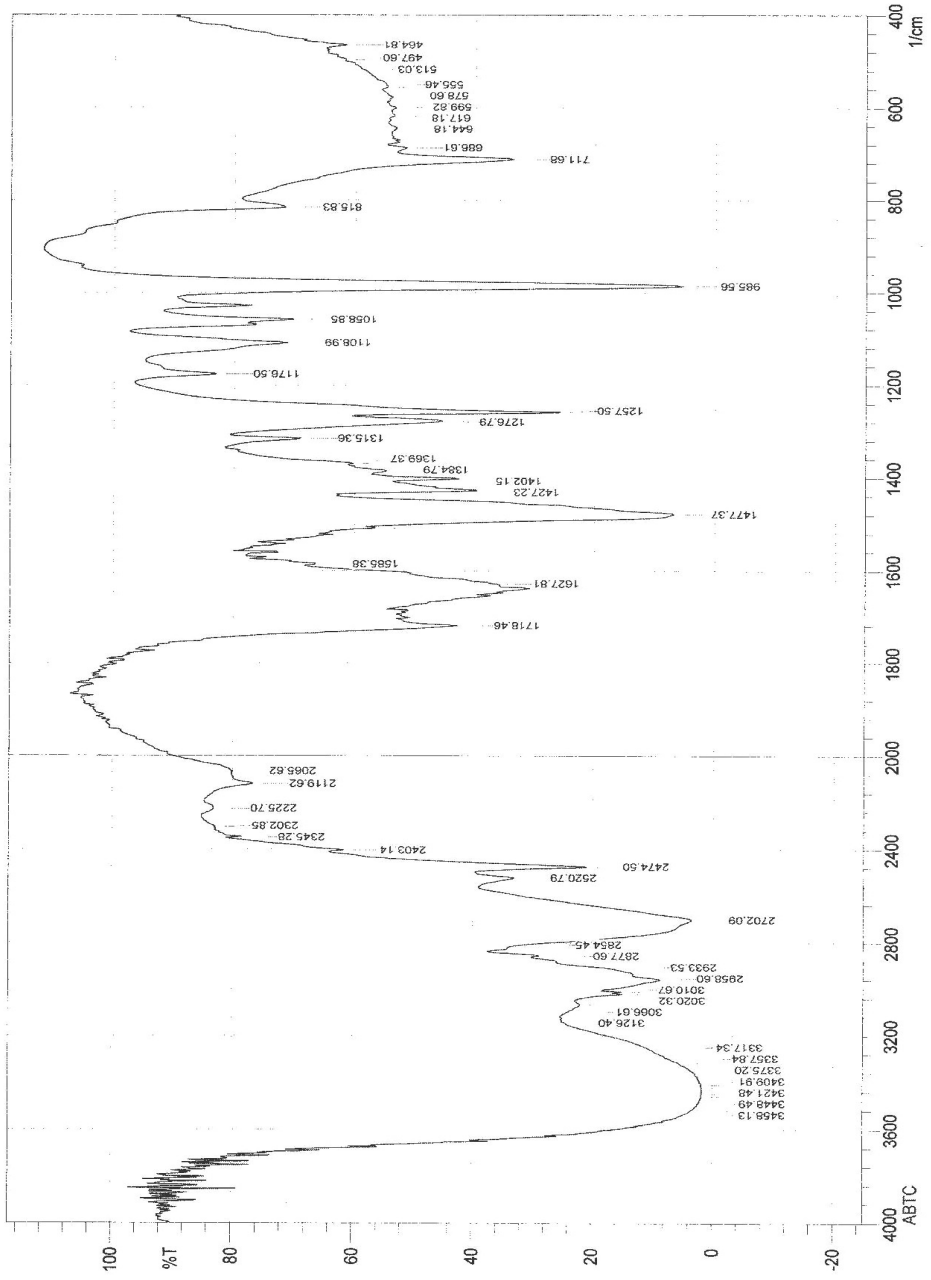


Figure.3 ^{1}H NMR spectrum of ABTC

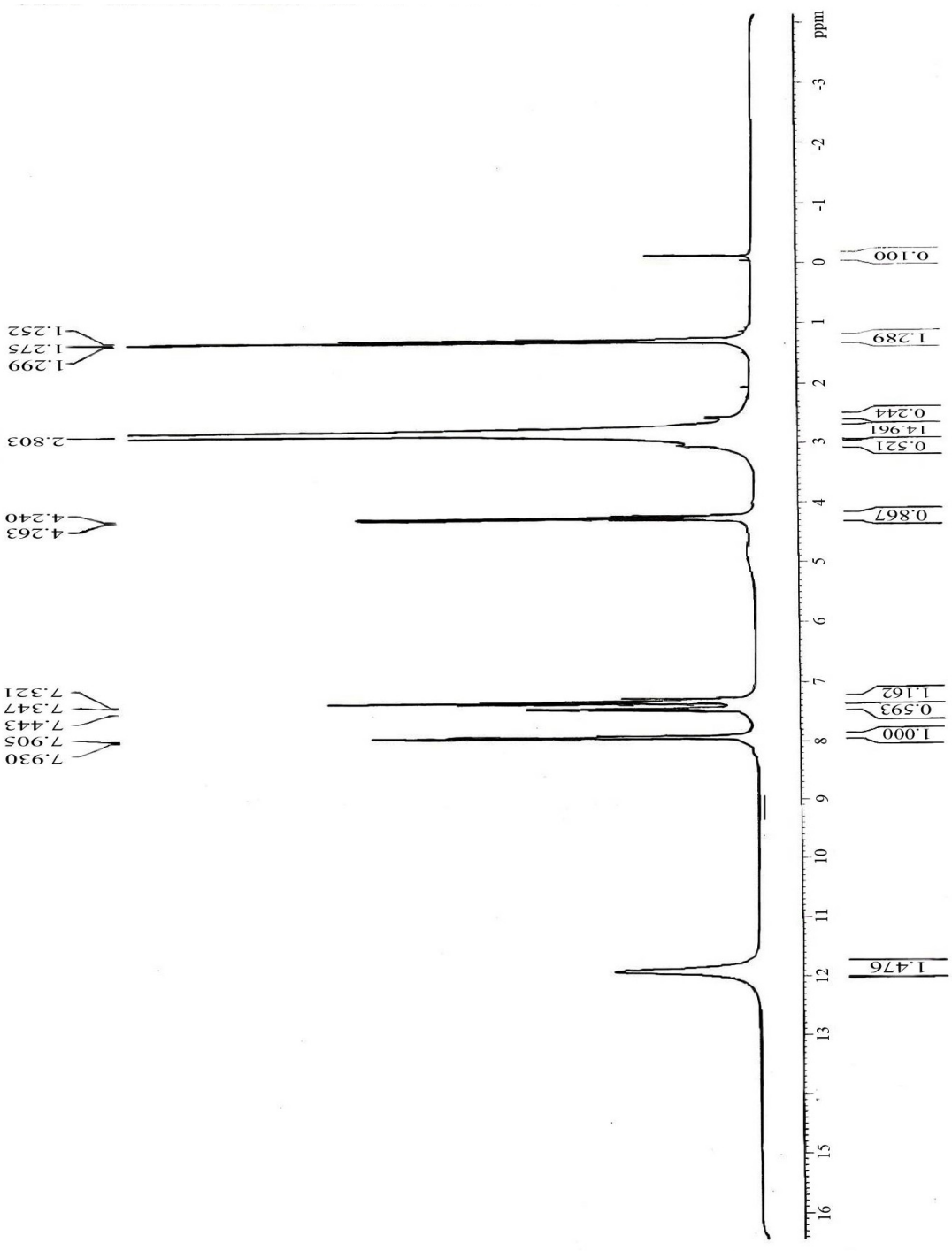
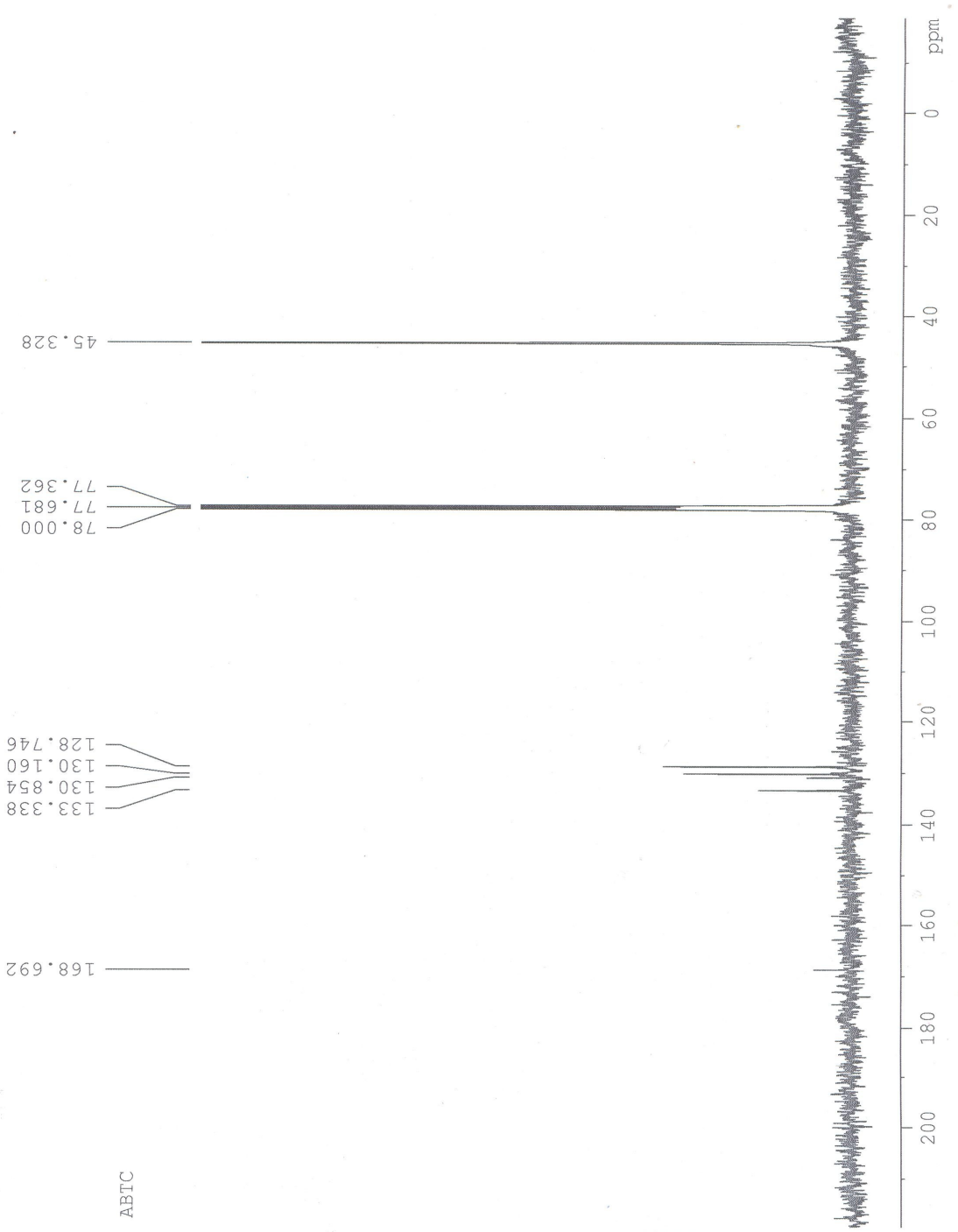
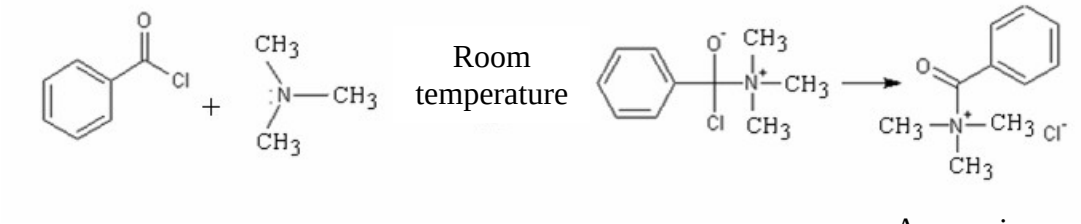


Figure.4 ^{13}C NMR spectrum of ABTC



Scheme.1



Benzoyl chloride Trimethylamine

Ammonium benzoyltrimethyl chloride

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