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Synthesis and Characterization of N-Benzhydrylbenzamide

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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, N-benzhydrylbenzamide was designed using computational techniques. This N-benzhydrylbenzamide was synthesized using Gabriel synthesis and Scotten-Baumann benzoylation reactions and characterized by FTIR and NMR spectroscopy. The FTIR spectrum of N-benzhydrylbenzamide shows the presence of various functional groups such as amide, aromatic groups and stretching vibrations for benzene derivatives. In addition, the ^{1}H NMR spectrum of N-benzhydrylbenzamide confirmed the presence of amide and phenyl group hydrogens whereas the ^{13}C NMR spectrum shows the presence of aromatic carbons and carbonyl group carbon. As the N-benzhydrylbenzamide structurally mimics CD40L, it can be considered as a candidate molecule for the further development of novel immunotherapeutic agent.

Key Words: N-Benzhydrylbenzamide, CD40L, Small molecules, Mimetic molecules.

INTRODUCTION

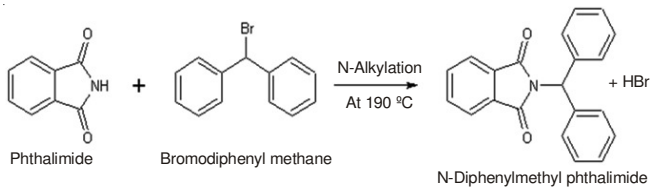
The CD40 receptor is a type I membrane protein that belongs to the nerve growth factor gene family. The CD40 ligand (CD40L), also known as T-B activating molecule, TNF-related activation protein or gp 39, is a 33 kDa type II membrane glycoprotein expressed on the surface of activated CD4+ T cells[1-6]. 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], CDK4[26], matriptase[27], Bcl-2[28], adenovirus protease[29] tyrosine kinase 2[30] and synthetic agonists of cytokine receptors[31,32].

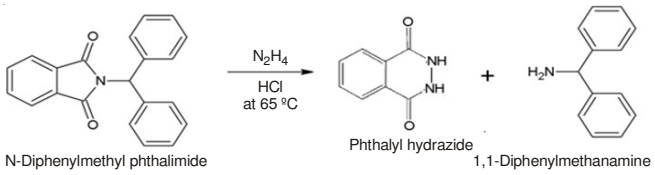
Using computational methods and the structural information of CD40-CD40L complex, a small molecule *i.e.*, N-benzhydrylbenzamide (NBB) was designed[33]. This small molecule mimics the active site of CD40L. N-Benzhydrylbenzamide was synthesized in the laboratory using organic synthesis methods and was characterized by chemical tests, FTIR and NMR spectroscopy.

EXPERIMENTAL

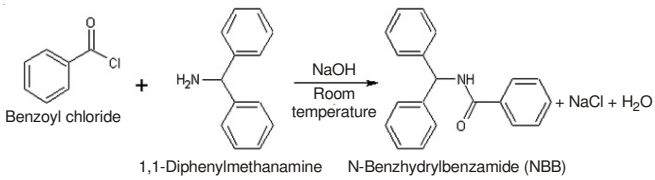
Synthesis of N-benzhydrylbenzamide: N-Benzhydrylbenzamide was synthesiszed in three steps. In step 1, phthalimide was subjected to N-alkylation to get N-diphenylmethyl phthalimide. In step 2, N-diphenylmethyl phthalimide was subjected to Gabriel synthesis to obtain 1,1-diphenylmethanamine. In the final step, Schotten-Baumann benzoylation of 1,1,-diphenylmethanamine was carried out to produce NBB. All the chemicals required for the synthesis of this compound were purchased from Sigma Aldrich, USA. The three reactions involved in the synthesis of NBB are presented in the **Schemes I, II** and **III**.



Scheme-I: Synthesis of N-diphenylmethyl phthalimide



Scheme-II: Synthesis of 1,1-diphenylmethanamine



Scheme-III: Synthesis of N-benzhydrylbenzamide

Synthesis of N-diphenylmethyl phthalimide: 76 g (0.55 mol) of finely powdered anhydrous potassium carbonate and 147 g of phthalimide were ground together in a glass mortar. The mixture was transferred to a round bottomed flask and treated with 284.4 g of bromodiphenyl methane. It was heated in an oil bath at 190 ºC under a reflux condenser for 3 h. While the mixture was still hot, the excess of bromodiphenyl methane was removed by steam distillation. N-Diphenylmethyl phthalimide commenced to crystallize near the end of the steam distillation. At this point, the mixture was cooled rapidly with vigorous swirling, so that the solid was obtained in a fine state of division. The solid was filtered with suction on a Buchner funnel. It was washed well with water and drained as completely as possible. It was then washed once with 60 % ethanol and drained again.

Purification of N-diphenylmethyl phthalimide: N-Diphenylmethyl phthalimide was taken in a beaker. Acetic acid was added and then the mixture was heated and stirred to dissolve the compound. The addition of acetic acid was stopped when the solution appeared clear. The solution was filtered with a fluted filter paper. Then it was set on the bench top and left undisturbed. After a while, clear crystals appeared in the beaker.

Synthesis of 1,1-diphenylmethanamine: An alcoholic suspension of 156.6 g of finely powdered diphenylmethyl phthalimide was warmed with 25 g of 100 % hydrazine hydrate. A white gelatinous precipitate was produced rapidly. The latter was decomposed (when its formation appears complete) by heating with excess of hydrochloric acid on a steam bath. The phthalyl hydrazide was collected, which was separated by suction filtration and it was washed with water. The filtrate was concentrated by distillation. It was cooled and filtered from the small amount of precipitated phthalyl hydrazide. It was rendered alkaline with excess of sodium hydroxide solution and the liberated 1,1-diphenylmethanamine was extracted with ether. The ethereal solution was dried with potassium hydroxide pellets, the solvent was removed using rotary evaporator and finally the residue was distilled. The 1,1-diphenylmethanamine was collected.

Synthesis of N-benzhydrylbenzamide: 1 mL of 1,1-diphenylmathanamine was suspended in 20 mL of 5 % sodium hydroxide solution in a well-corked boiling tube or small conical flask. 2 mL of benzoylchloride (0.5 mL at a time) was added with constant shaking and cooling in water (if necessary). It was shaken vigorously for 5-10 min until the odour of benzoylchloride disappeared. It was made sure that the mixture has an alkaline reaction. The solid benzoyl derivative (NBB) was filtered off. It was washed with a little cold water.

Purification of N-benzhydrylbenzamide: N-Benzhydrylbenzamide to be recrystallized was taken in a beaker. To that hot ethanol was added. It was swirled to dissolve the solid. The beaker was placed on the steam bath to keep the solution hot. When the solid was all in solution, it was filtered with a fluted filter-paper. Then it was set on the bench top and left undisturbed. After a while, clear crystals appeared in the flask.

Thin layer chromatography: Silica gel was used as an absorbent for TLC. 1:1 ratio of ethanol and acetic acid was used as a mobile phase. Since the sample to be studied was an organic compound, iodine vapours were used to stain the TLC plates. It is based upon the observation that iodine has a high affinity for both unsaturated and aromatic compounds. To 100 mL wide mouthed jar (with cap), a piece of filter paper and few crystals of iodine were added. Iodine has a high vapour pressure as a solid and the chamber will rapidly become saturated with iodine vapour. TLC plate was inserted and allowed to remain within the chamber until it developed a light brown colour over the entire plate. The TLC plates were removed carefully at this point and the spots were gently circled with a dull pencil, the iodine will not remain on the TLC plate for long periods of time, so circling these spots would be necessary to refer to these TLC's at a later date.

Chemical test for the identification of benzamide functional group in N-benzhydrylbenzamide: 1 g of the N-benzhydrylbenzamide was treated with a few drops of concentrated sulphuric acid and water. A white cloudy precipitate was formed. It was filtered, dried and its crystalline nature was checked.

FTIR spectroscopy: FTIR spectroscopic analysis of intermediate compounds N-diphenylmethyl phthalimide and 1,1-diphenylmethanamine and the final compound NBB was carried out to analyze different functional groups present in the compounds. FTIR spectra (ν_{max} in cm^{-1}) of these three compounds were recorded on a spectrophotometer (Thermo

scientific, USA) using KBr pellets. These FTIR spectra were obtained using the in-built Omnic software.

Nuclear magnetic resonance spectroscopy: The ^{1}H and ^{13}C NMR analyses of NBB were performed to confirm the presence of functional groups. The ^{1}H and ^{13}C NMR spectra were recorded on a (Brucker, USA) NMR instrument using $CDCl_3$ as solvent (chemical shift in δ ppm) and TMS as internal standard. These ^{1}H and ^{13}C NMR spectra of NBB were obtained using the in-built Topspin 2.0 software.

RESULTS AND DISCUSSION

N-Benzhydrylbenzamide was synthesized in three steps. The step 1 product, N-diphenylmethyl phthalimide is a white crystalline compound with pungent odour (yield 72 %, m.p. 107 ºC). Diphenylmethyl phthalimide was further characterized by FTIR spectroscopy. The step 2 product, 1,1-diphenylmethanamine was found to be a yellow coloured liquid with fishy odour. It was also further characterized by FTIR spectroscopy.

The percentage yield of the final product, NBB was 72 %. It is a white, amorphous and odourless powder. The m.p. of the compound was found to be 113 ºC. N-Benzhydrylbenzamide was found to be insoluble in water, but it is soluble in hot ethanol and chloroform. The N-benzhydrylbenzamide was checked for purity by TLC. Functional groups were confirmed by chemical tests. FTIR and NMR spectroscopy were performed to identify the molecular nature of the NBB. Results of these tests are presented below.

Purity by thin layer chromatography: The R_f value for the NBB was found to be 0.83 which is different from the R_f values of the reactants, benzoylchloride with R_f value 0.67 and 1,1-diphenyl methanamine with R_f value 0.73. The difference in the R_f values confirms the presence of the NBB. Moreover, the NBB preparation showed no contaminating 1,1-diphenylmethanamine and benzoylchloride, suggesting a pure preparation of NBB.

Identification of benzamide functional group: The melting point of the crystalline product formed by treating 1 g of NBB with a few drops of concentrated sulphuric acid and water was found to be 121 ºC. This ensures that the precipitate obtained is benzoic acid. This confirms that the NBB is a benzamide.

FTIR spectroscopic analysis: The FTIR spectrum of N-diphenylmethyl phthalimide shows the presence of functional groups such as amide at 3307 and 3191 cm^{-1}, aromatic carbon-hydrogen stretching at 3062 and 3047 cm^{-1}, alkyl carbon-hydrogen stretching at 2852 cm^{-1}, carbon-oxygen stretching at 1774 and 1733 cm^{-1} and a aromatic ring vibration at 1467 cm^{-1}. The FTIR spectrum of 1,1-diphenylmethanamine shows the presence of functional groups such as primary amine at 3446 and 3373 cm^{-1}, aromatic carbon-hydrogen stretching at 3082 cm^{-1}, alkyl carbon-hydrogen stretching at 2956 and 2858 cm^{-1} and asymmetric bending at 1623 cm^{-1}. The FTIR spectrum of the final product *i.e.*, N-benzhydrylbenzamide shows the presence of functional groups such as amide at 3396 cm^{-1}, aromatic carbon-hydrogen stretching at 3029 cm^{-1}, aromatic ring vibrations at 1602 and 1488 cm^{-1}, alkyl carbon-hydrogen stretching at 1452 cm^{-1}, C-O stretching at 1072, 1039 and 1016 cm^{-1} and stretching vibrations for benzene derivative at 871, 804 and 788 cm^{-1}.

NMR spectroscopic analysis: The ^{1}H NMR spectrum of NBB shows the presence of functional groups such as amide at 8.117 and 8.142 ppm and phenyl groups at 7.254, 7.420, 7.481, 7.506, 7.596, 7.620 and 7.644 ppm.

The ^{13}C NMR spectrum of NBB shows the presence of aromatic carbons at 127.541, 128.448, 128.651, 129.652, 129.291, 130.272, 132.710 and 133.777 and carbonyl group carbon at 172.332.

Conclusion

The FTIR spectrum of N-benzhydrylbenzamide shows the presence of various functional groups such as amide, aromatic groups and stretching vibrations for benzene derivatives. In addition, the ^{1}H NMR spectrum of N-benzhydrylbenzamide confirmed the presence of its functional groups such as amide and phenyl groups whereas the ^{13}C NMR spectrum shows the presence of aromatic carbons and carbonyl group carbon. As this compound, N-benzhydrylbenzamide mimics the binding site of CD40L; it will be subjected to binding studies and functional assays such as B-cell proliferation assay and antibody isotype switching to test the biological activity of this CD40L mimetic molecule.

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