



New Azo Amide Compounds Derived from Benzocaine Synthesis, Characterization, and Study of Biological Activity

Adeel Asghar* and Hussain Ali

Islamia University of Bahawalpur, Pakistan



Abstract

Benzocaine (0.05 mol, 7.42 g) was decomposed in NaNO_2 in the presence of HCl at temperatures ranging from 0 to 5 degrees Celsius to produce several novel derivatives of Azo compounds (50 mL). 2-Nitrophenol (80%, 7.5 mL) and compound 1 (0.05 mol, 10.67 g) were heated for two hours at reflux in 50 mL of ethanol. When the procedure was stopped, the mixture had cooled to a normal temperature. Filtered and water was used to clean the precipitate. The ethanol yield solidified. This reaction was examined using Thin-Layer Chromatography (TLC). Melting points, elemental analyses, FTIR, $^1\text{H-NMR}$, and $^{13}\text{C-NMR}$ were used to characterize all novel compounds. The antibacterial activity of these derivatives was also established, as was the anti-breast cancer activity of three of these compounds.

Keywords: Benzocaine; Azo Compounds; Amide Compounds; Anticancer and Anti-Microbial

Introduction

Drug formulation and design play a significant role in the search for novel medications. Due to repeated usage, many medications lose their effectiveness, necessitating a greater dosage or the substitution of the active ingredient. Since many anesthetics can lead to addiction and subsequent drug abuse, they are a distinct class of medications.

The process of designing and synthesizing innovative medications is time-consuming, costly, and labor-intensive. Additionally, the potential drug candidate must pass a vast array of tests in which every facet of potential chemical and biological components must be carefully considered.

A group ($-\text{N}=\text{N}-$) in their structure distinguishes azo compounds, which are among the most significant colored organic compounds. Because they prevent the formation of proteins and RNA, azo compounds have a wide range of uses. They also have medical benefits because they are anti-bacterial and anti-cancer, and they are used in cosmetics. Many illnesses are caused by microbes; hence, there is a significant amount of research on the subject of examining the biological activity of chemicals on different kinds of pathogenic bacteria. There are a growing number of infections caused by bacteria that have become resistant to most of the antibacterial medicines that are now accessible [1-3]. Several studies have been conducted on azo compounds against two species of bacteria that produce many diseases in humans: Bacteria that are Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*).

Benzocaine, an analgesic, is a simple chemical substance that relieves pain. It is utilized in topical, cutaneous, and mucosal formulations, but it is not effective in parenteral administration due to its low water solubility. As with any drug, benzocaine has both advantages and disadvantages; thus, novel derivatives having the benzocaine component as a substructure are always of relevance [4]. Additionally, benzocaine can be used to treat a variety of ailments, including toothaches, insect bites, rashes, and itching [5]. Benzocaine has several crucial characteristics that make it an effective drug, including a low pKa value (2.6) and aqueous solubility (0.4 mg/ml) [6].

The present study is designed to develop New azo amide compounds derived from benzocaine synthesis, characterize them, and study their biological activity.

Experimental Section

All ingredients were of the highest purity and were provided by Merck, Sigma-Aldrich, and

OPEN ACCESS

*Correspondence:

Adeel Asghar, Islamia University of
Bahawalpur, Pakistan,
E-mail: decentboys867hh@gmail.com

Received Date: 09 Aug 2026

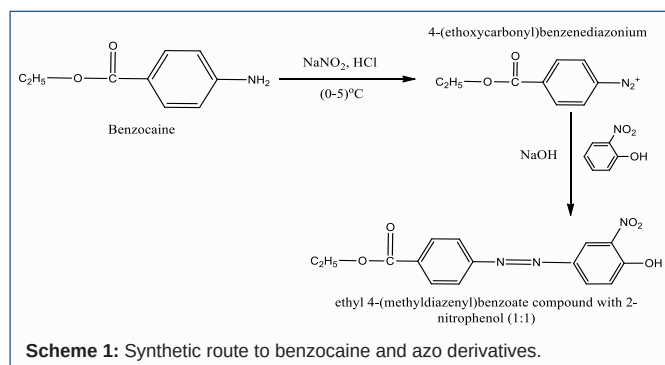
Accepted Date: 16 Sep 2026

Published Date: 18 Sep 2026

Citation:

Asghar A, Ali H. New Azo Amide
Compounds Derived from Benzocaine
Synthesis, Characterization, and Study
of Biological Activity. WebLog J Amino
Acids. wjaa.2026.i1804. [https://doi.
org/10.5281/zenodo.23104519](https://doi.org/10.5281/zenodo.23104519)

Copyright© 2026 Adeel Asghar. This is
an open access article distributed under
the Creative Commons Attribution
License, which permits unrestricted
use, distribution, and reproduction in
any medium, provided the original work
is properly cited.



Fluka. The melting points were determined using a Bauchi melting point apparatus B-545 (Bauchi Labortechnik AG, Switzerland, France). Data were collected microanalytically using a Vario Elementary instrument (Shimadzu, Japan). A Shimadzu Fourier Transform Infrared Spectrophotometer (Model 270) was utilized to record the IR spectra. NMR spectra were taken on 400 MHz (^1H) and 100 MHz (^{13}C) spectrometers (Tehran, Iran) using TMS as the internal standard and on a scale in ppm. Thin Layer Chromatography (TLC) was done on silica gel, and spots were observed using Iodine vapors. Analytical-grade reagents were utilized, and the solvents were filtered before use (Scheme 1).

General procedure for synthesis azo compound

At temperatures ranging from 0 to 5 degrees Celsius, benzocaine (0.06 mol, 7.42 g) was digested in NaNO_2 in the presence of HCl (50 mL). While stirring, a solution of benzoyl chloride (0.05 mol, 6 mL) was added dropwise to the liquid. This process takes place in a chilly environment and is chilled by an ice bath (approximately 5-10°C). The mixture was stirred for 45 minutes. The debris on the ground was filtered and cleaned with cold water. Water washes away any excess benzoyl chloride from the solid. (1) resulted from the crystallization of ethanol.

Compound 1 (0.04 mol, 10.77g) and 2-nitrophenol (%80, 7,5 mL) were heated in 50 mL of ethanol under reflux for 2 hours. After the reaction was stopped, the mixture was cooled to room temperature. Water was used to filter and wash the precipitate. The ethanol yield crystallized.

4(ethoxy carbonyl) benzene diazonium

Yield was 50% with a melting point of 148-150°C; color: yellow; FT-IR (KBr disc) (cm^{-1}): 3088 (C-H, aromatic), 1708(C=O), 1632(C=C, aromatic ring), 1316 (CH_3) and 823 (*p*-substituted Ar); $^1\text{H-NMR}$ (CD_3OD): 7.17-7.57(aromatic), 6.98-7.21(HC=CH of maleimide), 6.32-6.55 (HC=CH), 2.35 (CH_3) ppm; $^{13}\text{C-NMR}$ (CD_3OD): 164.9 (C=O), 134.5 (HC=CH of maleimide), 128.1-120.7 (C=C, aromatic), 19.9 (CH_3) ppm.

Ethyl 4-(methyl diazenyl) benzoate compound with 2-nitrophenol (1:1)

Yield was 84%; m.p. 182-184°C; color: yellow; FT-IR (KBr disc) (cm^{-1}): 3481 (O-H), 3108 (C-H, aromatic), 1705 (C=O), 1601 (C=C, aromatic ring) and 828 (HC=CH of maleimide); $^1\text{H-NMR}$ (CD_3OD): 6.78-7.48 (aromatic), 6.90-7.15 (HC=CH of maleimide), 6.31-6.58 (HC=CH) ppm; $^{13}\text{C-NMR}$ (CD_3OD): 170.8 (C=O), 157.4, 134.3 (HC=CH of maleimide), 128.2-112.0 (C=C, aromatic) ppm.

Antimicrobial Evolution

The newly synthesized chemicals were chosen for their

antibacterial properties. *Staphylococcus* (gram-positive) and *E. coli* (gram-negative) were utilized to select the most powerful compounds by using the agar diffusion technique [7]. 5 mg of each chemical was dissolved in one milliliter of Dimethyl Sulfoxide (DMSO), then diluted to 25 ml with distilled water to yield a 500 g/mL concentrate ion. The bacteria were grown on Muller histone agar media, which was incubated at 37°C for 24 hours. The efficacy of the investigated drugs was matched to DMSO, with the zone of inhibition assessed using a ruler [8-14].

Materials and Methods

Methyl Thiazolyl Tetrazolium (MTT) solution

To generate a 2 mg/ml quantity of the dye, 0.2g of methyl thiazolyl reagent (Bio-World, USA) was added and was diluted in 200 ml of PBS. To eliminate any blue formazan product, the solution was filtered through a 0.2-μm syringe filter and stored in sterilized, darkish lock vials at 4°C. The remedy was put to use within two weeks of its manufacture.

Cytotoxicity assay

Using 96-well plates (Santacruz Biotechnology, USA), MCF7 Breast cancer human cells were plated at 10000 cells/well, and 250 l of cells in fermentation medium were injected into each well of a sterilized 96-well microtitration plate.

The discs were covered with self-adhesive film, a lid was placed on top, and they were incubated at 37°C.

The media was withdrawn after 24 hours or when the cells were in exponential development, and successive dilutions of the extraction were added to each well at two-fold increased serial dilutions (dilution of the stock to 1/10 ml). For each, triplicates were used. Control cells were simply given serum-free media. The plates were then re-incubated at 37°C for 72 hours.

After 72 hours of exposure, cell growth was determined by draining the medium, adding 27 l of a 1.0 mg dose of MTT (Bio-World, USA), and incubating for 1 hour at 37°C. After discarding the MTT liquid, the crystalline material in the reservoirs was solubilized using 140 l of DMSO (Dimethyl Sulfoxide) (Santacruz Biotechnology, USA), accompanied by a 15-minute agitation at 37°C with shaking.

The absorbance was measured using a microplate reader (Biochrom, UK) at 585 nm (the test wavelength); the experiment was repeated five times.

The following endpoint characteristics were computed for each cell line:

1. Percentage of cellular metabolism or proportion of cellular proliferation (PR) = average of therapy / mean of the reference
2. The lowest concentration is capable of killing 50% of cells (LC50).

Cell culture for Normal embryonic cells

The Rat Embryonic Fibroblast (REF) cell line was grown in RPMI-1640 media with 10% FBS, 100 units/mL antibiotic, and 100 g/mL streptomycin and incubated at 37°C. The Iraqi Biopharmaceutical Company, Cultured Cells Lab, Baghdad, Iraq, provided all of the cell lines.

These cells are frequently evaluated for growth-accounting properties and certified.

Using the MTT cell viability assay, the anticancer efficacy of the synthesized derivative was assessed *in vitro* vs. a mammary gland MCF-7 cancer cell line.

Results and Discussion

In the current study, benzocaine was employed as a filler core component in the development of new Azo compound derivatives with a yield of 66-76%. The synthesized reactions are summarized in Scheme 1.

Characterization

The FT-IR spectra for both compounds show that C=O is absorbed at 1702 cm^{-1} , alkene group (HC=CH) absorbance at 3119 cm^{-1} , and bands corresponding to 1589 , 1600 , and 1512 cm^{-1} for aromatic rings. A H-X group at 3481 cm^{-1} and a M-yl group at 1316 cm^{-1} were also observed for both compounds (Figure 1).

Without a doubt, both the compounds named I and II were created. The existence of the azo in the $1630\text{--}1575\text{ cm}^{-1}$ range verified the effectiveness of the synthesis. Furthermore, the azo group's connection to the ortho position of the benzene rings was shown by the o-substituted benzene rings absorption at $750\text{--}775\text{ cm}^{-1}$.

The compounds were discovered using ^1H and ^{13}C NMR. The protons followed a similar trend ($-\text{CH}_2$). 4 protons showed as multiples in the $0.22\text{--}1.89\text{ ppm}$ range, while ($-\text{CH}_2\text{--CO-}$) proton signals emerged as multiples in the $1.43\text{--}2.87\text{ ppm}$ range. ($-\text{NH-CO-}$) emerged as a singlet in the range $1.04\text{--}3.67\text{ ppm}$ of amino acid subunits that are oriented by the functional group. The amino acid protons and other protons were thoroughly examined.

Biological activity

Bacterial community control is necessary to avoid disease, illness, degradation, spoilage, and pollution caused by them. The newly synthesized chemicals were tested *in vitro* for antibacterial efficacy against microorganisms. According to the antimicrobial activity that resulted, the majority of the investigated substances had moderate to large increases in productivity. The most efficient molecules are evaluated *versus* *S. aureus* in contrast to *E. Coli*, with 10 having the maximum activity compared to other variants. When these compounds were compared to the reference compounds (DMSO), we discovered that they exhibit antibacterial activity that is higher or nearly equivalent to theirs.

The strains of bacteria's sensitivity to the substances were measured using the Micro-well dilution method values for Minimum Inhibitory Concentration (MIC). From 12-hour broth cultures, bacterial strain inocula were made, and the suspensions were changed to have an acceptable turbidity of 0.5 McFarland. Compounds that were soluble in DMSO were first produced at the greatest concentration required for evaluation (200 g/mL) in order to achieve a concentration range of 6.25 to 200 g/mL . After that, 15 mL of successive two-fold dilutions were prepared. Sterile test tubes containing nutritious broth 95 L and 5 L for broth culture and inoculum were distributed into each well to create the 96-well plate plates, respectively. As a positive control, 250 L of nutritional broth without inoculation was placed into the first wells. The second well was filled with aliquots (100 L) of the 200 g/mL stock solution.

Each serial dilution was poured 100 L into five separate wells. The final well on each strip contained 5.0 L of the inoculum and 195 L of nutritional liquid without compound as a negative control. Before being incubated for 24 hours at the right temperature, the contents of

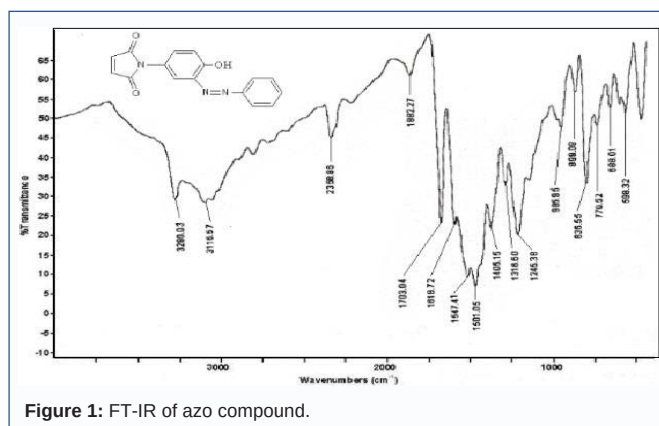


Figure 1: FT-IR of azo compound.

each well were agitated on a plate shaker at 350 rpm for two minutes. The amount of microbial growth in each medium was measured at 630 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc., Highland Park, Vermont, USA). The results were validated by plating 5 L of samples from clear wells on nutritional agar medium. The MIC was established as the smallest chemical concentration necessary to prevent microbiological growth. Ampicillin served as the microbiological reference standard for positive sensitivity.

In general, the anticancer potential of the synthesized chemical was higher ($\text{IC}_{65} = 450\text{M}$) after 62 hours.

Conclusions

In this study, we documented the production of various derivatives from benzocaine reacting with certain NaNO_2 and HCl to produce Azo compound derivatives. These compounds were discovered to be active against positive bacteria but not against gram-negative bacteria. The study proved to be a good inhibitor of anti-cancer cells. These derivatives were confirmed by spectrum data analysis, which included FT-IR, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, and elemental composition.

References

1. d'Alnoncourt RN, Csepei LI, Hävecker M, Girgsdies F, Schuster ME, Schlögl R, et al. The reaction network in propane oxidation over phase-pure MoVTaNb M1 oxide catalysts. *J Catal.* 2014; 311: 369-385.
2. Aljamali NM. The Various Preparation Methods in Synthetic Chemistry. 1 Edt. Evincepub Publishing House. 2019.
3. Aljamali NM. Effect of Conditions and Catalysis on Products. Eliva Press. 2021.
4. Paun A, Zarafu I, Caproiu MT, Draghici C, Maganu M, Cotar AI, et al. Synthesis and microbiological evaluation of several benzocaine derivatives. *Comptes Rendus Chimie.* 2013; 16: 665-671.
5. So TY, Farrington E. Topical benzocaine-induced methemoglobinemia in the pediatric population. *J Pediatr Health Care.* 2008; 22: 335-339.
6. Porasso RD, Bennett WD, Oliveira-Costa S, Cascales JL. Study of the Benzocaine Transfer from Aqueous Solution to the Interior of a Biological Membrane. *Biophys Chem.* 2009; 113: 9988-9994.
7. Abdul-Reda NA, Abass AK, Gebur NA. Synthesis, characterization, physical and biological properties of some cholesterol derivatives. *Eur J Chem.* 2016; 7: 206-212.
8. Strichartz GR, Sanchez V, Arthur GR, Chafetz R, Martin D. Fundamental properties of local anesthetics. II. Measured octanol: buffer partition coefficients and pK_a values of clinically used drugs. *Anesth Analg.* 1990; 71: 158-170.

9. Pinto LMA, Yokaichiya DK, Fraceto LF, de Paula E. Interaction of benzocaine with model membranes. *Biophys Chem.* 2000; 87: 213-223.
10. Yu HY, Sun P, Hou WY. Prolonged local anesthetic effect of bupivacaine liposomes in rats. *Int J Pharmaceut.* 1998; 176: 133-136.
11. Patai S. The chemistry of diazonium and diazo groups. Part 1. Wiley. 1978.
12. Nour A, Suad S. Synthesis, Characterization and Thermal Analysis for New Amoxil Ligands. *Asian J Chem.* 2019; 31: 1022-1026.
13. Aljamali NM. *Alternative Methods in Organic Synthesis.* 1th-Edition. Eliva Press. 2020.
14. Chattopadhyay SK, Sil S, Mukherjee JP. Synthesis of 2-aminosuberic acid derivatives as components of some histone deacetylase inhibiting cyclic tetrapeptides. *Beilstein J Organic Chem.* 2017; 13: 2153-2156.