IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 5, November 2026
Impact Factor: 8.2

Synthesis, Characterization, and Biological Evaluation of Substituted 1,5-Benzodiazepines: An Evidence Synthesis and Validation Framework for Fifteen Analogues

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Abstract : *The 1,5-benzodiazepine nucleus provides a versatile platform for the synthesis of diamine–carbonyl cyclocondensation products and subsequent biological evaluations. Claims regarding the successful synthesis of 1,5-benzodiazepines or their potential to act as antimicrobial agents must be supported by adequate structural and assay-level validation. This research combines validated literature reports of the synthesis of 1,5-benzodiazepines and their biological properties, with a critical assessment of a 15-member set of symmetrically diaryl-substituted analogues (C1–C15) described within a doctoral dissertation focused on pharmaceutical chemistry. Without assuming that the dissertation's reported yield and antimicrobial activity values were based upon direct observation, the molecular identity of each analogue was determined, the stoichiometric material requirements for each analogue were calculated, the process metrics for each analogue were established, and a multi-step analytical and biological validation protocol was developed. For example, for the C1 analogue design, the theoretical product mass would be 312.416 mg per 1.00 mmol of the limiting diamine reactant and the stoichiometric atom economy for the analogue would be 89.7%. If we assume that the dissertation's explicitly reported 88% yield was obtained, then the reaction mass efficiency would be 73.8% and the reaction-stage, solvent-free E-factor would be 0.40. However, these calculations are hypothetical and do not represent experimental measurements. A representative microbial inhibition matrix showed that analogues C7 and C10 were equivalent in terms of inhibiting fungal growth. While analogue C10 had the lowest assigned minimum inhibitory concentration (MIC) for Staphylococcus aureus, there was no experimental evidence available to establish which analogue was superior in terms of inhibiting bacterial growth. Therefore, this work establishes a clear distinction between literature-based evidence, chemical calculations, hypothetical data, and the experimental data necessary to provide publication-grade evidence of the synthesis of 1,5-benzodiazepines and the determination of their biological efficacy.*

Keywords: 1,5-benzodiazepines, cyclocondensation, structural characterization, antimicrobial susceptibility, minimum inhibitory concentration, structure–activity relationship, green chemistry, experimental reproducibility

I. INTRODUCTION

The seven-membered diazepine ring that is attached to a benzene ring produces many different isomers based upon the location of the nitrogen atoms within the rings. For example, in the 1,5-isomer, the two heteroatoms are located at ring position 1 and ring position 5, respectively. Therefore, we can't assume that the 1,5-isomers will behave biologically in a similar manner as the commonly known 1,4-benzodiazepine drugs. One of the most popular ways to create 2,3-

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dihydro-1H-1,5-benzodiazepines has been by reacting a vicinal aromatic diamine with two equivalents of an enolizable ketone. Curini et al. created 2,3-dihydro-1H-1,5-benzodiazepines through solvent-free reaction with either cyclic or acyclic ketones using ytterbium triflate as a promoter. Pozarentzi et al. developed a microwave-assisted cyclocondensation method using acetic acid to promote the reaction [1] [2].

The reasons behind why researchers would want to develop drugs that are effective against various pathogens is different than the purpose of developing synthetic methods to produce these drug molecules. For example, Wang et al. investigated the effects of heterocycle-substituted 1,5-benzodiazepines as potential treatments for both fungal and bacterial infections; while Caiana et al. examined the ability of several hydroxylated chemicals to act as antioxidants and to inhibit the growth of certain types of fungi [3] [4]. These studies demonstrate that modifications made to a molecule can affect how effective that molecule will be at inhibiting the growth of pathogens. However, simply modifying a molecule so that it has the same basic structure as another molecule does not guarantee that the modified molecule will be effective at inhibiting the growth of pathogens. Whether or not a particular molecule will be effective at inhibiting the growth of pathogens is dependent upon the type of organism being tested, the medium used to grow the organism, the number of organisms initially added to the medium, the method used to assess whether or not the organism grows, and how much of the chemical is available to the organism. There are standard methods available for assessing the susceptibility of pathogens to chemicals. For instance, CLSI M27 is used to determine the susceptibility of yeasts to chemicals, while CLSI M07 is used to determine the susceptibility of aerobic bacteria to chemicals [5] [6].

The fifteen 2-methyl-2,4-diaryl analogs (C1-C15) in the original dissertation were conceptually designed using 1,2-diaminobenzene and a singly substituted acetophenone which was employed twice. In addition to being conceptual designs, the abstract and methodology sections of the dissertation state that the comparative optimization, physical properties, and antimicrobial matrices were modeled, as opposed to experimentally verified [7]. Therefore, the issue at hand is not if the molecules have been synthesized or tested for activity, but how to differentiate the effects of molecular design, stoichiometry calculations, literature precedents, and experimental confirmation.

The contributions made by this work are: (i) the creation of a trackable chemical identity and reaction mass framework for C1-C15 compounds; (ii) the development of a comparative analysis of reported experimental methods used in synthesis, biology, and validation studies; (iii) the formulation of a replicable sequence of characterization-to-bioassay testing; and (iv) the quantitative auditing of model outcomes and process metrics, but without displaying these audits as measured data. This article is a critical narrative synthesis of existing evidence and a suggested validation framework, rather than a report of new synthetic or microbiological research.

II. LITERATURE REVIEW

A. Cyclocondensation and catalyst development

In Italy, Curini et al. (2001) studied how ytterbium triflate catalyzed the reaction that occurs when o-phenylenediamines react with cyclic or acyclic ketones [1]. Curini et al. reported that this reaction yielded high amounts of product when conducted without solvents and established an effective way to use Lewis acids to form the 2,3-dihydro skeleton. This research provides support for the types of reactions studied here; however, it does not confirm that 90 °C, 60 minutes or acetic acid are applicable to the entire library of compounds from C1-C15. Additionally, because there were several different kinds of ketones used in Curini et al.'s work, it illustrates why researchers need to report substrate scope rather than assume it is the same as the reaction itself.

In their study on solvent-free, microwave-driven conversion using an acetic acid catalyst, Pozarentzi et al. (2002), at the University of Thessaloniki, investigated the use of o-phenylenediamines and ketones to produce rapidly and efficiently prepared compounds with isolated-product characterization [2]. This work provides direct evidence of the potential of acetic acid as a promoter; however, due to different forms of heat transfer, this work does not provide quantitative data that would allow us to estimate how much energy we can expect from a conventional thermal reaction based on what has been observed in microwave reactions.

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Jarikote et al., (2003) of the National Chemical Laboratory in Pune, studied ionic-liquid mediated synthesis at room temperature [8]. The 1,3-di-n-butylimidazolium bromide medium they used allowed for cyclocondensation to occur without any of the traditional catalyst being added; this demonstrates how critical medium effects are as well as the potential for ionic stabilization. However, it cannot be concluded that the ionic liquid has environmental advantages over other types of solvents because there are so many factors to consider such as recovery, purification burden, waste generated and cost.

De and Gibbs (2005) reported on the use of scandium (III) triflate as a catalyst under solvent-free conditions, which provided an additional Lewis-acid pathway and claimed to have developed a reusable catalyst [9]. Similarly, Kumar et al. (2006) utilized silver nitrate as part of a solvent-free methodology [10]. Collectively, these two articles demonstrate that other forms of Lewis acids can also be effective activation agents. Additionally, they raise questions regarding the comparative aspects of the remaining metal content in the final products; the quality of the products produced; the extent to which the catalyst is lost during the process; and whether or not the catalyst performs similarly regardless of whether it is applied to electron-rich or electron-poor aryl methyl ketones.

The study by Kuo et al. (2008) in Taiwan examined 2,4,6-trichloro-1,3,5-triazine-catalyzed condensation of 1,2-diamines and enolizable ketones [11]. This study demonstrates how inexpensive and easy-to-handle reagents can be activated at relatively mild temperatures and pressures, while focusing on isolating the final product. Additionally, it shows why a researcher cannot simply use a single substrate to support their claim that a reaction is applicable generally.

The authors, Jadhav & Kim (2013) in the Republic of Korea, evaluated heterogeneous silver-substituted silicotungstic acid as a solid acid catalyst for a variety of reactions conducted in a solvent-free ambient environment [12]. The authors reported an average isolated yield of 88% within 40 minutes for all evaluated reactions and the ability to reuse the solid acid catalyst for five consecutive cycles. Jeganathan & Pitchumani (2014) evaluated the use of HY zeolite as a solid acid catalyst at 50 °C and were able to reuse the zeolite as a solid acid catalyst for six consecutive cycles [13]. Both of these solid acid catalyst studies represent similar evaluations of low-solvent processes as opposed to the biologically modified analogue; however, the cost of producing solid acid catalysts, scaling up solid acid processes, and separating solid acid catalysts from product streams will be greater than if the evaluation had focused solely on reaction times.

The use of supported catalysts has increased in popularity over the years. This can be seen in Kusuma et al.'s (2022) development of ferrocene-anchored activated carbon as a catalyst for a "one pot" synthesis of benzodiazepines. Zhang et al. (2023) developed a magnetic cerium-based dual-acid nanocatalyst for the synthesis of lactam-fused 1,5-benzodiazepines. They synthesized 20 examples with 85-98% yields using their specified reaction parameters. Gharehkhani et al. (2026) utilized a graphene-oxide/tannic-acid network nanocatalyst for the synthesis of 4-substituted analogues. Although these studies indicate that researchers are continuing to develop new catalysts, they modified different parts of the scaffold and used different reaction partners than those that will be used in the present study. Therefore, the numerical yields reported in these studies cannot be used as an indicator of how successful the reactions would be with the bis(acetophenone) C1-C15 derivatives.

B. Characterization and biological evaluation

The researchers in the Wang et al. (2015) study from Hebei Normal University synthesized 36 different compounds containing both a 1,5-benzodiazepine ring system and either thiophene or thiazole rings with ester functional groups [3]. The researchers characterized each compound using various analytical techniques such as IR, proton/carbon NMR, MS and elemental analysis. In addition to these characterization studies, the authors also obtained single crystal x-ray diffraction data for one of the synthesized compounds. The researchers evaluated the activity of the compounds against four microorganisms (Cryptococcus neoformans, Candida albicans, Escherichia coli and Staphylococcus aureus) and found that many of the heterocycle-containing analogues exhibited very low minimum inhibitory concentration (MIC) values for their anti-fungal properties. While the authors' biologically active compounds have similar substitutions to those reported here, they differ significantly in terms of substitution pattern (i.e., position) relative to the C1-C15

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positions. Therefore, it is not possible to copy their MIC values directly. However, what can be taken away from their research is that there are alternative methods available to confirm identity and to evaluate susceptibility towards specific microorganisms.

The research group at Hebei Normal University (An et al., 2016) synthesized additional modified 1,5-benzodiazepines containing either pyridine or phenyl rings, as well as ester groups; characterized the newly prepared compounds using infrared spectroscopy, nuclear magnetic resonance spectroscopy, mass spectrometry and elemental analysis; and evaluated the biological activity of these compounds against bacteria [17]. This study provides another directly applicable comparison of the structure-activity relationships among various appendages and their respective locations on the 1,5-benzodiazepine ring system. The authors point out that modifying the appendages attached to the 1,5-benzodiazepine skeleton and/or changing where they are attached to that skeleton will invalidate a direct transfer of a single-variable SAR from a symmetrical 2,4-diaryl series to a asymmetrical 2,4-diaryl series.

Caiana et al. (2021) in Journal of the Brazilian Chemical Society, developed a synthesis for hydroxylated benzodiazepines and evaluated their antioxidant properties as well as their ability to kill or inhibit growth of the Sporothrix schenckii complex [4]. The authors' most successful hydroxylated compound is not within the exact C1–C15 structural design; however, it has other types of modifications. While this research provides evidence for the activity of hydroxylated C4 compounds against Cryptococcus or Candida, it does not provide definitive proof. Rather, it suggests that further studies are needed to determine the solubility characteristics of these compounds, analyze their spectral data and evaluate their efficacy in controlling fungi.

The use of 1,5-benzodiazepines as separate syntheses to evaluate for potential anti-cancer effects and possible combinations with methotrexate was examined by Gawandi et al. (2021) in human cell lines [18]. Similarly, Chen et al. (2014) investigated 1,5-benzodiazepine-2,4-dione derivatives as potential anti-cancer agents [19]. Both studies provide valuable insight into the diverse pharmacological possibilities of the 1,5-benzodiazepine scaffold; however, neither study supports the claim that the proposed set of analogs possess anti-fungal or anti-bacterial activity due to their distinct biological targets and carbonyl architecture.

In addition to the previous study, Floyd et al. (2024) systematically evaluated several variables associated with microdilution assays conducted in 384-well plates for both Candida albicans and Cryptococcus neoformans. These included testing different types of media, incubation times and readouts [20]. The results indicated that the ranking of active compounds can vary greatly depending on how the assay is designed; therefore, it is essential to determine if the assay meets quality control requirements prior to evaluating compound activity. Additionally, Balouiri et al. (2016) discuss complementary methods for determining susceptibility using both agar diffusion and broth dilution techniques as well as the limitations inherent in interpreting the data obtained via each method [21]. Therefore, this literature supports establishing defined endpoints, concentration ranges and control groups prior to estimating the MIC from the zone size of growth on an agar plate.

C. Evidence synthesis

The literature has shown how to create a viable pathway to the 1,5-benzodiazepine scaffold, as well as showing that there are some independently created derivatives which show biological activity. The literature does not show that the exact fifteen proposed symmetrical bis-aryl analogue compounds were isolated at the stated yields, nor that the source's numerical MIC matrix was created through experimentation. When the proposed mechanisms for halogen polarizability, electronic substitution and ortho crowding are removed from solubility, purity and repeated assay considerations; they remain testable hypotheses. Therefore, the current research gap is centered on providing documentation and comparison of syntheses of each of the fifteen designed compounds and organism specific biological data for the designed compounds.

Table I provides examples of previously reported synthetic approaches used with the current class of substrates, along with limitations regarding the extent of application (or transferability) of each strategy.

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TABLE I. SELECTED VERIFIED SYNTHETIC PRECEDENTS

Study	Demonstrated approach	Relevance / qualification
Curini 2001 [1]	Yb(OTf)3; solvent-free	Lewis-acid precedent
Pozarentzi 2002 [2]	AcOH; microwave; neat	Energy mode differs
Jarikote 2003 [8]	Ionic-liquid medium	Medium and recovery matter
Jadhav 2013 [12]	AgSTA, ambient; 5 cycles	Reported reuse; different catalyst
Jeganathan 2014 [13]	HY zeolite, 50 °C; 6 cycles	Heterogeneous reference
Kusuma 2022 [14]	Supported ferrocene catalyst	Recent process innovation
Gharehkhani 2026 [16]	GO hybrid nanocatalyst	Different derivative class

III. RELATED WORK

The closest synthetic comparison can be made to the acetic acid promoted cyclocondensation of ketones and diamines reported by Pozarentzi [2] as both methods utilize an inexpensive, easily obtainable activator and similar carbonyl-containing reactants. However, the microwave conditions used in Pozarentzi's studies, as well as the isolation and analytical characterization of the product mixtures obtained from those experiments, should not be equated to the conventional thermal model presented in this dissertation. Other alternative catalytic systems for the same reaction are reported in the literature including Curini's ytterbium method [1] and Jeganathan & Pitchumani's HY-zeolite method [13]. Comparisons of the reported isolated yield data for these methods will require that each system be evaluated based on the specific substrate(s) studied, the purity requirements established, the scale of operation and the limits of work-up boundary conditions employed in each study.

The most closely related biological citations include Wang et al., and An et al. [3] [17]; while each study combined multi-technique chemical characterization with organism-specific evaluations, the heterocyclic and ester modifications of each study created two distinct chemical types. Caiana et al. [4] provided a helpful precedent for a hydroxylated analog; however, the organism panel used in that study was not compatible with the four-organism panel proposed herein. In comparison to the previously cited studies, the C1-C15 source had an identifiable identity design and proposed testing plan, yet did not provide independently archived chromatograms, spectra, isolate mass and plate readings.

The next table (Table II) compares the closest synthetic and bioassay methods to the missing evidence for the fixed analog series..

TABLE II. CLOSEST WORK AND UNRESOLVED DIFFERENCE

Approach	Verified evidence	Gap relative to C1–C15
AcOH/microwave [2]	Isolated products; spectral analyses	No matched thermal-run data
HY zeolite [13]	Published catalyst reuse	Different acid and process boundary
Wang biological series [3]	Synthesis, multi-modal spectra, MIC	Different chemotype; no C1–C15 MIC
Caiana hydroxyl analogues [4]	Synthesis and Sporothrix assays	Different organisms and positions

There is no evidence to support that there is a gap in demonstrating that the synthesis of these bis-aryl compounds has been performed as part of a batch-linked process, with quality control of the purity of the compounds, and that the comparative microbiological studies have been conducted on them. Therefore, instead of making claims about a new lead or a universally optimized reaction, the proposed framework establishes evidence gates.

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IV. MATERIALS AND METHODS / PROPOSED FRAMEWORK

A. Research design and provenance

The research described here is a structured critical narrative synthesis based on the M.Pharm. dissertation provided, as well as being cross-referenced with publisher, PubMed and standards records. The studies included in the analysis were selected based upon their direct relationship to chemical routes, close compound characterization, similar biological end-points and comparable reporting requirements for analytical methods. This is not a systematic review; therefore, there has been no extensive database-wide searches (and subsequent exports), deduplication logs, independent dual screenings, or risk of bias assessments performed. The literature-reported numerical data has not been pooled as there is a difference in substrate identity, assay methods, and responses reported.

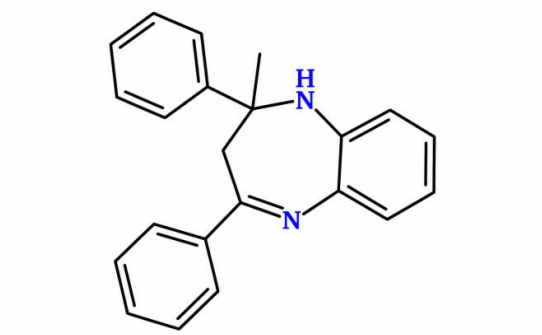
Five levels of evidence will be maintained throughout: E1 is based on a study that was independently published, E2 is based on a chemical/mathematical determination from given inputs, E3 is based on the explicitly stated model values within the dissertation, E4 is based on a proposed laboratory protocol which should be executed and E5 is based on a measurement which is currently unavailable in the record. Table III serves as the interpretation key for all following results.

TABLE III. EVIDENCE PROVENANCE CATEGORIES

Level	Example	Scientific status
E1	Published catalyst or MIC study	Literature reported; different samples
E2	Formula and stoichiometry	Calculable from structures
E3	C1–C15 yields and MICs	Illustrative/modelled only
E4	Selected AcOH thermal protocol	Proposed, unvalidated
E5	Original spectra, actual MIC plates	Not supplied

B. Analogue definitions and reaction model

C1-C15 are designated to be 2-methyl-2,4-diaryl-2,3-dihydro-1H-1,5-benzodiazepines. In addition to being the same kind of molecule, each of the pendant aryl groups carries the same substituent. Figure 1 displays the C1 core reference structure, which has the RDKit connectivity representing the neutral composition C22H20N2. While the identity of the aryl substituent(s) and their positions change for C2-C15, the basic diazepine structure remains constant. Positional-isomer assignments require direct experimental evidence, and the stereochemistry of the possible stereogenic C2 position is not indicated.



2-methyl-2,4-diphenyl-2,3-dihydro-1*H*-1,5-benzodiazepine (C1)

Fig. 1. Original structural depiction of the C1 reference connectivity; a calculated target, not a measured structure.

The general formula for substituting an acetophenone, Ar-CO-CH3, can be represented as (1). In (1), D stands for o-phenylenediamine; K stands for substituted acetophenone; and P stands for the desired 1,5-benzodiazepine. The letters

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used to designate the molecules in (1) are the same as those used to designate the mass of each molecule in all subsequent formulas. At the proposed 1.00 mmol scale, the limiting reagent is the diamine. At this scale, the charge of ketone is 2.20 mmol, and acetic acid is 0.20 mmol (20 mole percent). The theoretical product mass is determined by the molecular formula and limiting-reagent stoichiometry. This dissertation has chosen to conduct this synthesis at 90°C for 60 minutes without using a bulk reaction solvent, however, it does not provide an authenticated run log [7].

$$D + 2K --> P + 2H2O \qquad (1)$$

The first step of the mechanism is carbonyl activation, then condensation, then iminium/enamine transformation, and finally seven membered ring formation. It is important to note that the mechanistic arrow shown here are simply hypothesis for how this thermal reaction could occur, and they do not represent kinetic evidence. Each sample must be isolated, dried and weighed; both crude yield and purity corrected isolated yield must be reported individually.

The entire proposed evidence generation process is shown in figure 2. At this time, we have only completed design and calculation of each of the experiments.

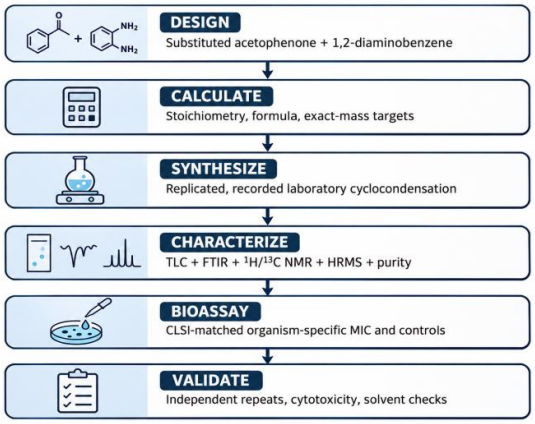

Fig. 2. Proposed evidence-generation workflow; all stages following design/calculation require real laboratory execution.

C. Identity, purity and physicochemical verification

A unique labeling of the synthesis batch, dating of all reagents used, isolation of a dry mass, and analysis of the purity of the sample are required prior to claims regarding the antimicrobial activity of any analogue. An acceptable minimum characterization of the analogue would include FTIR showing the loss of the starting carbonyl group and the presence of other relevant functional groups; proton and carbon NMR spectra that support the assignment of ring methylenes, methyls, aromatics, and substituted carbons; high-resolution mass spectrometry confirming the elemental composition; and another method of determining the purity of the sample. A single TLC spot does not provide sufficient evidence of purity due to the possibility of co-elution. Chemical formulas and nominal masses do not allow for discrimination between ortho, meta, and para positioning isomers.

Elemental analysis or an alternative chromatographic method should provide supplementary data to the primary data set. If there is ambiguity about the ring regiochemistry or stereochemistry of the molecule, then either a two-dimensional NMR assignment or crystal structure determination may be required. Raw spectra, acquisition conditions, solvent used, instrument calibration information and processed peak tables should be archived. A predicted spectral region does not equal a measured peak, and a calculated [M+H]+ signal is not equal to an acquired high resolution mass spectrometry (HRMS) signal.

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DOI: 10.48175/IJARSCT-41079

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D. Antimicrobial assay validation

The organisms to be tested will include C. neoformans, C. albicans, S. aureus, and E. coli. Development of yeast testing is based on CLSI M27, as well as the procedural control organisms specific to each of those tests. Dilution tests for aerobic bacteria are based on CLSI M07 [5] [6]. Each test must use authenticated strains of the organisms. Additionally, each test must include appropriate growth and sterility controls. Each reference drug tested must have its quality control results fall into acceptable limits. All two-fold dilutions performed must be verified. The solvent concentration used in the test must match the vehicle control. Each test must occur on separate days. Because there can be issues with color or solubility when reading the results of yeast tests, verification of precipitation, blank subtraction, and identification of an alternate viability readout are required. Floyd et al.'s work in 384-well formats illustrates how readout choice affects yeast tests [20].

The MIC is the lowest concentration that meets the defined end-point. It is essential to record the "end-point rule" and "off-scale values" instead of interpolating. Growth inhibition, as well as transport and diffusion, can be seen in agar inhibition zones. Zone diameters should not be used in place of MIC. To determine if an agent is fungicidal or bactericidal, it will take separate survival/subculture studies [21]. Before a biological lead can be nominated, a selectivity metric based on a defined mammalian cell type must be established.

E. Quantitative and environmental calculations

The theoretical Atom Economy (AE) of C1 does not include excess ketone and catalyst. However, Reaction Mass Efficiency (RME) includes charged reactants and depends upon the actual amount of product that was isolated. M denotes molar mass, m denotes mass, and letters p, d, k, and a denote product, diamine, ketone, and acetic acid, respectively. Mp is the mass of the isolated product. The Reaction-Stage E-Factor below includes the catalytic acetic-acid charge, and excludes work-up solvent, wash water, and solvent recovery:

$$AE = 100\,Mp/(Md + 2\,Mk) \quad (2)$$

$$RME = 100\,mp/(md + mk) \quad (3)$$

$$E = (md + mk + ma - mp)/mp \quad (4)$$

A solvent-excluding E-factor should not be applied to say that the entire process is environmentally friendly. Sheldon has stated that there must be clearly defined boundaries for mass as well as complementary metrics for energy, hazards, and life cycles [22] [23]. Conversion of mass concentration (MICmass in µg/mL) to molar concentration (MICmol in µM) requires the molecular weight (MW) of the compound in g/mol, however, this will not enhance the ability to dilute.:

$$MICmol = 1000\,MICmass/MW \quad (5)$$

F. Planned inferential analysis

Planned studies will require independent synthetic batches for each design condition and independent antimicrobial assays for each day of testing. Replicate targets will be determined through a combination of power analysis and/or prospective precision estimates. Tabulations of isolated yields (with raw masses), method defined purities and run variabilities will be provided for all isolated yields. Minimal inhibitory concentrations (MICs) will be presented for each isolate, as well as biological repeats on a two-fold dilution scale. Censoring will occur at tested limits. Cytotoxicity will be based upon independently measured concentration-response curves. It should be decided prior to beginning the study if the comparisons will be organism specific or multi-organism. Statistical testing is not appropriate given the triplicates created in this current data set.

V. IMPLEMENTATION AND EVALUATION PLAN

A useful center for designing an initial design of experiments with the C1 model has been identified as follows; it is not a validated optimum: 1.00 mmol diamine, 2.20 mmol substituted acetophenone, 0.20 mmol AcOH, 90 °C, and 60 minutes. The C1 model will require 108.144 mg of diamine, 264.332 mg of acetophenone, and 12.010 mg of acetic

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acid, based upon the purity of the reagents and the verified density correction. All thermal equilibration, vessel geometry, mixing, and moisture levels should be recorded since the nominal heating-block temperature does not represent the true reaction temperature. Based upon the above, the theoretical mass of the product is 312.416 mg.

To validate, you should perform experiments using either a controlled factorial or response-surface experimental design to assess the effects of reaction times, temperatures, amounts of acetic acid and excesses of ketones. Do not change more than one variable at a time. Also, use a control that does not include a catalyst as well as a published and feasible comparative example. Separately report conversions and isolated and pure yields. You will need to apply this protocol to several types of substrates such as those with electron-donating, electron-withdrawing and/or ortho-substitutions prior to making claims about the general applicability of the protocol to a wide range of substrates. Your workup must account for all solvents used during extraction and recrystallization as part of your overall process mass intensity measurement.

TABLE IV. C1–C15 DESIGN INVENTORY; CALCULATED COMPOSITION

Code	Aryl R	Molecular formula	MW (g/mol)
C1	H	C22H20N2	312.416
C2	4-CH3	C24H24N2	340.470
C3	4-OCH3	C24H24N2O2	372.468
C4	4-OH	C22H20N2O2	344.414
C5	4-F	C22H18F2N2	348.396
C6	4-Cl	C22H18Cl2N2	381.306
C7	4-Br	C22H18Br2N2	470.208
C8	4-NO2	C22H18N4O4	402.410
C9	4-CN	C24H18N4	362.436
C10	4-CF3	C24H18F6N2	448.410
C11	4-NH2	C22H22N4	342.446
C12	3-Cl	C22H18Cl2N2	381.306
C13	3-OCH3	C24H24N2O2	372.468
C14	2-Cl	C22H18Cl2N2	381.306
C15	2-OCH3	C24H24N2O2	372.468

Table IV is where the dissertation's master compound list is located. Each of the formula listed above are supposed to represent the neutral products being proposed. However, no equipment used in this study has been able to confirm that each individual sample contains the chemical components described in its formula. In order for us to evaluate the structure-activity relationship between the chemicals being studied and the biological activity of those chemicals, we will need to have our structural drawings, analytical acquisition metadata and batch records all linked together under the same code prior to evaluating the structure-activity relationship.

VI. RESULTS: EVIDENCE AUDIT AND CALCULATED OUTCOMES

A. Deterministic stoichiometry

Using 108.144 mg of o-phenylenediamine with two molar equivalents of acetophenone (240.302 mg), the chemically balanced target mass is 312.416 mg and the water formed in the reaction is 36.030 mg. From equation (2), the atomic

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International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 5, November 2026
Impact Factor: 8.2

efficiency (AE) is 89.7%. By using a ketone charge that has 2.20 molar equivalents, the total mass of the reactants becomes 372.476 mg. For the purpose of illustration only, let us assume that the E3 model presented in the dissertation, which predicted an 88% isolated yield, applies here. This assumption will result in a calculated product of 274.926 mg. Therefore, the relative molecular efficiency (RME) is 73.8%, and the reaction-stage E-factor is 0.40. These calculations do not imply any balance, chromatogram, weighing, or actual conversion.

Table V presents the complete numerical mass balance, along with its associated evidence boundaries.

TABLE V. C1 CALCULATED MASS METRICS AND BOUNDARIES

Metric	Value	Evidence status / boundary
Formula mass	312.416 g/mol	E2 target C1
Diamine charge	108.144 mg	E2: 1.00 mmol
Acetophenone	264.332 mg	E2: 2.20 mmol
AcOH charge	12.010 mg	E2: 0.20 mmol
Modelled yield	88%	E3; not an observed mass
Conditional product	274.926 mg	E2 conditional on E3
Atom economy	89.7%	E2 stoichiometry
RME	73.8%	E2 conditional on E3
Stage E-factor	0.40	E2; work-up excluded

Fig. 3 compares the two mass-based percentage metrics; the plotted RME assumes the source model yield.

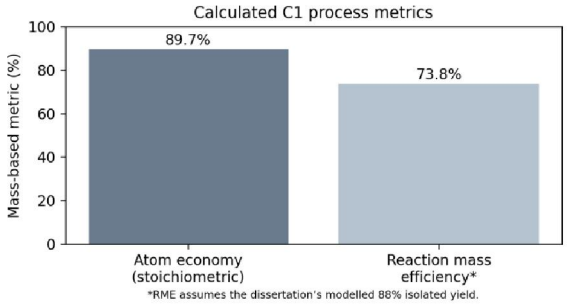

Fig. 3. Calculated C1 material metrics; RME depends on an explicitly modelled, unverified 88% yield.

B. Illustration-only antimicrobial matrix

Table VI maintains the exact four columns of MIC data from my dissertation for use as a model-to-model numerical interpretation. The MIC data, whether referenced agent entry in the source, is not authenticated MIC data and therefore does not provide evidence of susceptibility, biological potency, significance, selectivity or candidate drug. The table is used to show transparency in ranking criteria and a future blind testing strategy; it does not represent positive experimental results.

TABLE VI. ASSIGNED MODEL MICs ONLY (µg/mL); NOT MEASURED

ID / R	C.n.	C.a.	S.a.	E.c.
C1 H	64	128	128	128
C2 4-CH3	64	64	64	128
C3 4-OCH3	32	64	64	64

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DOI: 10.48175/IJARSCT-41079

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ID / R	C.n.	C.a.	S.a.	E.c.
C4 4-OH	16	32	32	64
C5 4-F	32	64	64	64
C6 4-Cl	16	32	32	32
C7 4-Br	8	16	32	64
C8 4-NO2	64	128	128	128
C9 4-CN	16	32	32	64
C10 4-CF3	8	16	16	32
C11 4-NH2	32	64	64	64
C12 3-Cl	16	32	32	64
C13 3-OCH3	32	64	64	64
C14 2-Cl	32	64	64	128
C15 2-OCH3	64	64	64	128

In the provided matrix, C7 and C10 each have 8 and 16 µg/mL assigned to C. neoformans and C. albicans. Of all the entries in the S. aureus row of the matrix, C10 was assigned the lowest value (16 µg/mL), while C6 and C10 tied for assigning the lowest value to E. coli (32 µg/mL). The 90% yield that was modeled by C2 is the highest value in the synthesis-yield column, however, this value was not experimentally obtained. These results reflect the data used to construct the illustration, however, no association with antimicrobials was empirically found.

Fig. 4 is intended to show the MIC grid that was assigned. It should be noted that this figure is not being used to illustrate an experimental response.

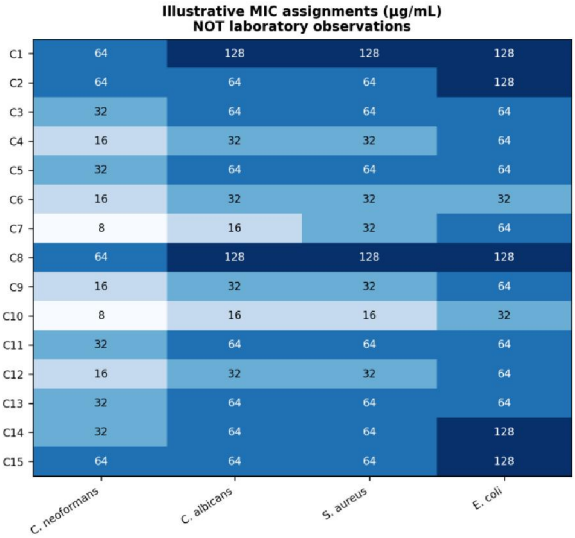

Fig. 4. Heatmap of dissertation-assigned hypothetical MICs. Darker cells indicate higher MIC; no value derives from microbial plate data.

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C. Provenance failures and verification gates

There is no signed sample ledger for C1-C15 provided by the source; there are no batch linked instruments used to identify products; no complete purity chromatography files exist; there are no authenticated assay plate images; no raw two-fold dilution readings were taken; there are no verified biological replicates; and there were no original quality control records for the reference drug. In addition, the simulated triplicate inhibition zone values do not represent separate experiments and should therefore never be used in calculating confidence intervals for experimental data, nor should they be used to determine p-values. As a consequence of the above-stated deficiencies, the only valid conclusion of this audit is that the ready-to-use status of the molecular designs and reaction calculations as a planning tool remains intact. However, the experimental design, the structural confirmation, and the biological validation remain unfulfilled.

Table VII operationalizes the additional experimental and documentary requirements necessary to prepare an empirical submission.

TABLE VII. MINIMUM DATA PACKAGE FOR SUBMISSION-READY EXPERIMENTAL PAPER

Stage	Essential record	Pass criterion
Synthesis	Batch notebook, isolated mass	Independent repeats with identity
Structure	Raw 1H/13C NMR, HRMS, FTIR	Assignments match structure
Purity	HPLC/UPLC or valid orthogonal QC	Predeclared purity threshold
Antifungal	M27-compatible plate files	Valid controls; repeated MIC
Antibacterial	M07-compatible plate files	Valid controls; repeated MIC
Safety	Cytotoxicity and exposure	Acceptable selectivity evidence
Sustainability	All solvent, water and waste masses	Clearly bounded PMI/E-factor

VII. DISCUSSION

The synthesis shows that there are two distinct ways to make inferences. On the one hand, if we consider only the chemical aspect, the stoichiometry of a 1:2 net mixture of an aromatic diamine with a symmetrically substituted acetophenone can be balanced; therefore, the target composition can be determined, and the approximate feed amounts can be calculated. On the other hand, at the applied or practical level, the success of the reaction and the structure of the resulting products cannot be inferred by simply using a balanced equation. To move from the suggestion of a potential cyclocondensation to a credible research claim in pharmaceutical chemistry, we must provide measurements of purity, verification of identity through several types of spectra, and an accounting of mass balance. Examples presented in the literature demonstrate that these tasks can be accomplished, however, none indicate that they were carried out for C1-C15 [1] [11].

Catalyst selection cannot simply be based on the hypothetically greatest possible yield. A solventless reaction may nevertheless necessitate the use of solvents during chromatographic purification or recrystallization; the introduction of an ionic liquid may create additional solvent recovery requirements; and supported/heterogeneous catalysts must demonstrate that they retain their catalytic activity through successive cycles. Therefore, comparisons made to Jadhav & Kim and Jeganathan & Pitchumani should motivate a holistic assessment of the entire process including work-up, reuse, energy, and impurities [12] [13]. It is important to remember that conditional C1 atom economy is a characteristic feature of the balanced reaction and not a determination of the environmental impact.

The C7/C10 MIC relation as shown in Figure 1, can only be seen as a characteristic of the assigned two-fold grid. To compare the effects of an aryl group symmetrically substituted, it would require that each analog had been tested under the same conditions (same strain of microbe, same media used to expose the analog to the microbe, same dissolution process for the analog, same analytical quality, etc.). Wang et al. studied several structurally related antimicrobials with experimentally determined results. These results make the scaffold biologically relevant; however, they do not prove

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that other models may be effective against the same microbes [3]. Until selectivity and independent replication of the dose-response relationship can be demonstrated for the compounds, organism-specific biological prioritization should continue. Additionally, C10 is considerably larger than C7 in terms of molecular weight. Therefore, if there is a relationship between the MICs based on molecular weight, this does not necessarily imply that both will have equal molar exposure (5).

The most direct immediate value of this work is its ability to report data in a way that provides some protection against failures (e.g., fixed compound codes; equations based on explicit mass inputs; literature comparator limits; and an assay gate-keeping sequence). A scientific paper based on the framework will have greater scientific validity when it includes a set of validated examples of the most common structural types, rather than when there are additional unsupported figures or precision created through the construction of replicates.

VIII. LIMITATIONS AND THREATS TO VALIDITY

This review is based upon a selection of literature rather than a systematic review (PRISMA compliant) and thus cannot make claims as to completeness or study specific risk of bias assessment. All formulas were taken directly from the dissertation, and each was independently checked for its C1 connectivity. They do not represent experimentally determined structures. The reported stoichiometric metrics were calculated using the nominal molecular weight and the nominal charge of the pure reagent as stated in the dissertation. The displayed E-factor does not take into account the effects of work-up. The 15-model MIC matrix and yield data presented are hypothetical. Any attempt to infer correlations or rankings of mechanisms based on these data will be completely confounded by the lack of experimental validation. There was no patients, animals, cell cultures, or laboratory data collected for this publication.

In addition, other possible external validity limitations include possible stereogenic centers; unmeasured tautomer or conformer population sizes; incomplete measurement of aqueous solubility; lack of organism strain identification within the generated data set; and reliance of assay endpoints on both the medium used and how results are read. The author and institution names found at the beginning of the header come directly from the front cover of the dissertation and will need to be verified prior to being published as an article in a peer-reviewed journal, original content permission, and to determine which journal would best publish this research. No missing email addresses have been created.

IX. CONCLUSION AND FUTURE WORK

Verified literature has documented multiple pathways to obtain substituted 1,5-benzodiazepines as well as demonstrated that certain independently characterized benzodiazepine derivatives possess antimicrobial properties and/or other biological activity. Although the attached C1-C15 dissertation presents a specified molecular structure and approximate stoichiometric calculations for a particular class of benzodiazepines, the C1-C15 dissertation primarily serves to provide examples of optimization, physical properties and MIC values. This manuscript will reconcile the evidence provided by the C1-C15 dissertation with other published evidence; calculate a C1 atom economy of 89.7% and conditional reaction mass metrics; and propose a staged analytical and microbiological protocol which will allow for the transformation of synthesized analogues into verifiable research observations.

Future research will need to: 1. experimentally create an example of each type of halogen-donating, strongly electron-withdrawing and ortho-substituted group; 2. duplicate isolated yields; 3. collect complete evidence for identity and purity; 4. confirm that strain-specific minimum inhibitory concentrations (MICs) are comparable to Clinical and Laboratory Standards Institute (CLSI) methods using authenticated controls; and 5. gather information on solubility, mammalian cytotoxicity, selectivity, solvent inventory, and catalyst reuse. Only after all of this has been done can lead optimization, predictive structure-activity relationship (SAR), or comparisons of green processes be made.

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