1.0. Introduction:
The rich structural diversity and complexity of natural products had always played a significant role in drug discovery. In some cases they display all the properties in the molecule required to become as drug while in some case they provide a platform which needs structural modulations to optimize pharmacodynamic, pharmacokinetic, safety properties as well as the enhancement of the biological activities of the lead structure.\(^1\) In the last 30 years, more than half of the molecules approved by FDA for the treatment of various diseases are either natural products or derived from them, while only 36% are considered as truly synthetic. Furthermore, this percentage of novel/inspired natural product molecules increases to 68% in case of anti-infectives (antibacterial, antifungal, antiparasitic, and antiviral compounds) and 79.8% in case of anticancer.\(^2\) Natural products have therefore served not only as precursors for semi-synthetic derivative libraries,\(^3\) but also inspired a significant interest in developing novel synthetic methodologies aimed at creating skeletal, functional and stereochemical diversity i.e. "natural-like" novel synthetic compounds.\(^4\) Thus, a common belief during the synthesis or semi-synthesis of structurally complex molecules is that, molecules similar to natural products can be better recognised by a therapeutically relevant protein molecule assembled from a series of biosynthetic enzymes.\(^5\) In the past, synthetic chemists have made substantial efforts in developing an arsenal of synthetic methodologies to generate the libraries of compounds containing structural complexity.\(^6\) Innovations in synthetic methodologies led to the generation of many techniques:


\(^5\) Mc Ardle, B. M.; Quinn, R. J. Chembiochem. 2007, 8, 788.

1. Combinatorial chemistry
2. Target-oriented synthesis
3. Functional-oriented synthesis
4. Diversity-oriented synthesis

In the past century combinatorial chemistry was the only dominating strategy in synthetic chemistry which allows the synthesis of plethora of compounds for biological screening. But the major problem associated with combinatorial chemistry has been that the compounds produced have a limited structural diversity. Since the strategy involves the rapid synthesis of chemical entities derived from all permutations and combinations of monomers used for synthesis; for example, 5 alcohols coupled to 5 carboxylic acids would lead to 15 ester products as the combinatorial set. Thus, this exercise can pass to the synthesis of large numbers of compounds, but structurally all the compounds would be close enough. This was also due to the reason that the structural diversity of the products was only due to the building blocks and starting scaffold which resulted in the same framework in every instance. To achieve the maximum structural diversity, efforts should include the variety in building blocks, functional group and stereochemistry and most importantly the high degree of variation in molecular frameworks.

In this regard diversity-oriented synthesis (DOS) has come forth as a persuasive tool in synthesizing structurally diverse small molecule libraries. DOS can be defined by the most commonly used statement as “Diversity-oriented synthesis involves the deliberate, simultaneous and efficient synthesis of more than one target compound in a diversity-driven approach to answer a complex problem” where the complex problems may include binding, catalysis, phenotypic effects, etc. The most common approach to introduce variety in small molecule libraries is through applying complexity-generating

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reactions to a diverse set of starting materials. In this context multicomponent reactions have seen a high tide in their application towards the rapid synthesis of structurally varied small molecule libraries from functionally diverse starting materials. They employ more than two starting materials and convert them directly into the product where most of the atoms of the starting materials are incorporated in the final product. The various advantageous associated with them include:

1. They are atom economic i.e., if not all then the majority of atoms of the starting materials are incorporated into the product.
2. They are highly efficient because they generally accompanied with one-step synthesis instead of multiple sequential steps.
3. They are highly convergent i.e., several starting materials combine in one reaction to form the product.
4. Exhibit a very high bond-forming-index (BFI) i.e., several non-hydrogen atom bonds are formed in one synthetic transformation.
5. High structural complexity can be introduced by combining structurally diverse starting materials.
6. The various functionalities of starting materials allow the post transformation of product to heterocyclic natural product like structures.

Several multicomponent reactions have been developed in recent past like Ugi reaction, Passerini reaction, Bogni reaction, 3-CR and Hantzsch

reaction. However, from diversity-oriented synthesis perspective, Ugi multicomponent reaction offers the widest scope combining four diversity (Ugi-4CR) components in a single step. Furthermore, the starting substrates involved in the reaction are readily available and contain functionalities that can undergo further subsequent transformations to provide a high degree of structural variation.

![Diagram of Diversity Oriented Synthesis through Ugi 4CR Reaction]

**Figure 1.** Four component Ugi reaction towards diversity-oriented synthesis.

Ugi reaction belongs to the category of isocyanide-based multicomponent reactions and was first reported by Ivar Ugi in 1959. The classic reaction results in the formation of α-N-acylamino amide as a core skeleton structure which mimics acyclic peptide-like compounds occurring in nature.

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**Classic Ugi Reaction:**

\[
R_1 R_2 + R_3 NH_2 + R_4 OH + R_5 N^2C \rightarrow R_1 R_2 R_3 R_4 R_5
\]

**Mechanism of the reaction:**

\[
R_1 R_2 + R_3 NH_2 \leftrightarrow R_1 R_2 N R_3 \leftrightarrow R_1 R_2 N R_3 R_4 OH \leftrightarrow R_1 R_2 N R_3 + R_4 O^-
\]

\[
R_1 R_2 N R_3 + C\equiv N + R_4 CO_2 \leftrightarrow R_1 R_2 N R_3 R_4 CO_2 \leftrightarrow R_1 R_2 N R_3 R_4 O R_5
\]

**Figure 2.** Classical Ugi-ICR reaction and its mechanism.

The widely accepted mechanism involves the condensation of oxo and amine components in the first step.\textsuperscript{18} The condensation leads to the formation of imine which acts as a more activated carbonyl analogue for the further progress of reaction. Since the acid component is available in reaction that protonates the nitrogen atom of imine and increases its electrophilicity, thus making the substrate more accessible for nucleophilic attack. In the next step, the carboxylic acid anion reacts with isocyanide carbon atom and makes it as a synthetic equivalent of vinylidene carbene which further reacts with electrophilic carbon of imine in S_N^2 fashion. The isocyanide intermediate thus formed undergoes intramolecular N-acyl transfer to give final stable Ugi product. The reaction is usually conducted in a polar protic solvent such as methanol. The mild reaction conditions allow the inclusion of a variety of functionality which can be further used up in concurrent reactions \textit{i.e.}, pre- and post-condensation modifications to yield a variety of heterocyclic compounds. These compounds can then serve as scaffolds for the synthesis or semi-synthesis of natural products, therapeutic agents and combinatorial libraries.

For example, Wang \textit{et al.}\textsuperscript{19} applied multicomponent Ugi reaction in the diversification of artemisinin, naturally occurring peroxide isolated from the \textit{Artemisia annua}, \textit{L.}

Artemisinin, and widely used for the treatment of malaria. Strategy involved the synthesis of two monomers of artemisinin containing aldehyde and amine at terminal position, which further reacts with isocyanides and different carboxylic acids to give a library of structurally complex artemisinin derivatives. Diversity in substrates also includes phenols as a substitute of carboxylic acids. The resultant products were tested for their anticancer potential and found to be active against panel of human breast cancer cell lines. This led to the identification of one artemisinin dimer showing IC$_{50}$ value of 12 nM on BT474 cell line, nearly 600 times more potent than artesunate, a semi-synthetic derivative of artemisinin used for the treatment of severe malaria.\(^\text{20}\)

![Chemical structures](image)

Figure 3. Diversity-oriented synthesis of artemisinin analogs through Ugi-4CR reaction.

In an attempt to bring out the diversity in the synthesis of a library of molecules, Stauffer and coworkers\(^\text{21}\) employed a broad range of substrates. Reaction strategy involved the high degree of variety in three substrates \textit{viz.} aldehydes, carboxylic acids and amines, while tert-butyl isocyanide was used as a fixed substrate. The obtained library was screened against severe acute respiratory syndrome coronavirus, where the most active compound was obtained after the reaction of 3-pyridyl carboxaldehyde, tert-butyl aniline, 2-furanoic acid and tert-butyl isocyanide. Since the reaction resulted in the formation of racemic product (IC$_{50} = 4.8$ \textmu M), therefore authors had resolved and then isolated the single enantiomer through chiral stationary phase supercritical fluid


chromatography. Out of two enantiomers only \( R \) isomer was active having \( IC_{50} \) of 1.5 ± 0.3 \( \mu M \) and also found to specifically inhibit 3CLpro versus PLpro.

![Chemical structures](image)

**Figure 4.** 3CLpro inhibitor by Ugi-4CR reaction.

Although peptide bond formation is one of the most studied C-N bond forming reactions throughout the history of organic synthesis, yet the tripeptide synthesis with a non-natural configuration and modified side chains of amino acids suffers with the lack of efficient synthetic methodologies. Furthermore, traditional coupling methods also suffer in synthesizing all the possible stereoisomers of tripeptide. In this regard, Ostaszewski *et al.*\(^{22} \) had used a novel Ugi 4-CR approach for the synthesis of all the eight possible stereoisomers of tripeptide aldehyde \( (Z)-L\text{-leu-L\text{-leu-L\text{-leu-H}}}) \) \( (Z=\text{benzyloxy carbonyl}) \) proteasome inhibitor (MG-132), while previously only two stereoisomers were reported. The authors first synthesised enantiomerically stable 2-isocyno-4-methylpentyl acetates as precursors for Ugi reaction which further reacts with enantiopure \( N\)-benzyloxy carbonyl leucines, 2,4-dimethoxybenzylamine and isovaleraldehyde to give the desired product in high yields. The obtained products were further functionalized to obtain terminal aldehyde of tripeptides. The synthesized compounds were shown to

exhibit antitumor activity and enhanced cytostatic/cytotoxic effects in chemo- and radiotherapy. It had also been found that stereochemistry of the final product significantly affect the biological activities where \((S, R, S)\-(-)\)-configured product is found to be 5-fold more potent than MG-132 in terms of inhibiting ChTL activity.

![Chemical structure diagram](image)

**Figure 5:** Tripeptide synthesis using Ugi-4CR multicomponent reaction.

Same group in 2011\(^\text{23}\) demonstrated the application of Ugi reaction in the synthesis of novel thioredoxin-thioredoxin reductase system inhibitors which are one of the attractive targets for anticancer therapies. The reaction strategy involved the incorporation of characteristic features such as an electrophilic fragment attached to peptide core which is found in the majority of known inhibitors of Trx system.\(^\text{24}\) They applied Ugi four components reaction for creating peptide linkage and 2-(2,6-bis(trifluoromethyl)benzoyloxy)methyl)acyrlic acid as a precursor for introducing electrophilic fragment to the desired molecule. The acid component reacts with isovaleric aldehyde, benzyl isocyanacetate and benzylamine to give the final product having IC\(_{50}\) value of 0.4 μM in Trx-TrxR insulin reduction assay. Also the compound was found to exhibit antitumor activity as well as a good inhibitor of NF-κB and AP-1 induction.

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Figure 6. Ugi reaction in the synthesis of novel thioredoxin-thioredoxin reductase system inhibitors.

In the same year Mangunuru and co-workers\textsuperscript{25} had explored Ugi 4-CR approach in the synthesis of a library of tripeptides and further explored them as gelators in different solvents. Strategy again involved the convergence of diverse functionalities of starting materials in a single entity through Ugi reaction. Several compounds from the library were found to be efficient gelators for ethanol aqueous and DMSO aqueous solutions. The compounds synthesised from formaldehyde, p-methoxybenzylamine, p-bromobenzoic acid and ethyl 2-isocyanooacetate, were found to be the best molecules having high efficiency of gel formation at 0.2 wt% in a DMSO-water (1:2) mixture.

Figure 7. Small molecular gelators through Ugi reaction.

Later, Ambra and co-workers\textsuperscript{26} reported the application of Ugi reaction in synthesizing molecules with a terminal acid functionality. The terminal acid group was then converted into hydroxamic acid which acts as a zinc binding group in histone deacetylase (HDAC) inhibition. A library of molecules was synthesized by varying all the four components of starting materials. All the molecules showed great potential in HDAC inhibition while the compound containing cyclohexyl isocyanide, phenyl ethanol,


benzyl amine and hydroxamic acid of pimelic acid with activity comparable to that of SAHA (IC₅₀ value of 1.2 μM).

![Chemical Structure]

**Figure 8.** HDAC inhibitors after the post-transformation of Ugi Products.

Ugi reaction can also be applied in tandem with other reactions to introduce diversity in products. Tandem reactions offer a particular advantage in the synthesis of structurally diverse compounds where the product is formed after a series of transformations and isolated only in the final step. For example Taleb et al. demonstrated the tandem use of Groebke-Blackburn and Ugi reactions in the synthesis of polysubstituted imidazopyridines and imidazopyrazines. The synthesized compounds were further tested for their anticancer potential, where some of the molecules were found to be highly potent in specific anti-breast cancer activity against MCF7 cell line, and others display specific effects against melanoma cancer cell line (M8). Also authors have shown the importance of imidazobenzothiazole framework in improving *in vitro* anticancer activities. The compounds were also tested for antibacterial properties where some of them showed significant potency against hospital-resistant clinical isolates, namely *E. coli, K. pneumontiae, S. epidermidis, P. aeruginosa, P. vulgaris* as well as against gram positive methicillin-sensitive and methicillin-resistant *S. aureus* bacteria.

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Recently, Rivera et al.,\textsuperscript{28} demonstrated the diversity-oriented synthesis of novel bis-spirostanic conjugates using different Ugi four-component reactions. In the reaction

\begin{figure}
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\includegraphics[width=\textwidth]{figure9}
\caption{Groebke-Blackburn and Ugi reactions in the synthesis of polysubstituted imidazopyridines.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10}
\caption{Diversity-oriented synthesis of novel bis-spirostanic conjugates using different Ugi four-component reactions.}
\end{figure}

strategy, first the steroidal skeletons with different functionalities were synthesized and then subjected to combinatorial principle through multicomponent Ugi-4CR with other precursors involved in the reaction. The selection of functionalities on steroidal skeleton was done on the basis of the strength of the chemical bond required for the stable ligation of spirostanic steroids. Also during the variation in amine and isocyanide, hydroxamic acid had been used as a substitute for carboxylic acid, resulting in 1,5-disubstituted tetrazole via an electrocyclic ring closure.

Tron et al.\textsuperscript{29} also used the strategy of employing multifunctional precursors in Ugi reaction for the synthesis of symmetrical and unsymmetrical bis-(\(\beta\)-aminoamides). Their reaction of secondary diamine, paraformaldehyde, water and pentyl isocyanide yielded no product due to the poor nucleophilicity of water. After trying with different acids as additives, the authors reported the efficient use of 2-hydroxymethyl benzoic acid as an acidic nucleophile to obtain the desired product. The reaction of piperazine with 2 equiv. of acid, benzyl isocyanide and paraformaldehyde in methanol at reflux temperature, gave the desired product in 90% yield. The Ugi product was further utilised in the synthesis of ranolazine, an anti-angina agent.

\[\text{Synthesis of Ranolazine}\]

\[\text{Figure 11. Ugi reaction for the synthesis of bis-(\(\beta\) aminoamides).}\]

Ugi reaction also finds application in introducing functional diversities in various heterocyclic core structures, thus making them more versatile precursor for various

chemical transformations as well as a tool for combinatorial approach in drug discovery. Jonny and coworkers\textsuperscript{30} described the applicability of Ugi reaction in creating skeletal, functional and stereochemical diversity in pyrazine-2(1H)-one based frameworks. Synthesis was done in tandem fashion where first phenylglyoxal, benzylisonitrile, benzylamine and a set of N\textit{Boc} protected amino acids were allowed to react till completion. After the consumption of starting materials, addition of trifluoroacetic acid resulted in the synthesis of highly substituted pyrazin-2(1H)-ones.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{reaction_scheme.png}
\caption{Ugi based synthesis of libraries of pyrazine-2(1H)-one.}
\end{figure}

The importance of introducing various functionalities in starting materials had been described by Hong \textit{et al.},\textsuperscript{31} with their work on indole-based heterocycles. In their report, they used indole-2-carboxaldehyde, propylamine, cyclohexyl isocyanide and iodine substituted benzoic acid for Ugi reaction, which after the formation of product can be converted into three distinct set of indole-based heterocycles by varying the reaction conditions.


conditions. The key step involved the C-H activation at various positions and thus cyclization to form rings of different size.

![Chemical structure image]

**Figure 13.** Ugi reaction in indole-based heterocycles.

Guchhait *et al.*,\(^{32}\) described the use of TMSCN as a synthetic equivalent of isocyanide in Ugi reaction. The reaction post Ugi transformation led to the synthesis of highly substituted heterocyclic frameworks. The authors used KF to activate TMSCN towards nucleophilic attack on heterocyclic amidine in the presence of water. The reaction circumvents the need of isocyanide, thus reduces its associated side reactions and resulted in high yield of products. In their next attempt they used bifunctional aldehydes which can undergo cascade reaction and gave structurally diverse scaffolds. In this regards they used methyl 2-formylbenzoates with various heterocyclic amidines to produce isoquinolinone-fused imidazoheterocycles, thus affording one-step 3-component 6-centre cascade reaction in water.

Figure 14. Diversity-oriented synthesis of imidazoheterocyclic structures through Ugi reaction.

Recently Sotelo et al.,\textsuperscript{33} had reported Ugi approach for the synthesis of libraries of structurally diverse quinoxalines. The reaction starts with the convergence of 2-oxoaldehydes, isocyanide, carboxylic acids and mono-\textit{boc} protected phenylenediamines in a single pot operation. The final product contains NHBoc group in its amine precursor which undergoes cyclization with oxo group of aldehydes precursor leading to the synthesis of quinoxaline. Reaction was also tried with phosphoric acid as a substitute of carboxylic acid, where reaction gave the desired product in good yields.

Figure 15. Ugi reaction in the synthesis of libraries of quinoxalines.

2.0. Aims and objectives:

The literature review makes it clear that Ugi reaction can offer access to structurally diverse compounds in a very efficient manner. Natural products having any of the four different functionalities \textit{i.e} carbonyl, amine, acid or isocyanide, can bring diversity with the wide range of other structural units available. Also literature shows the examples where first functionalities required for Ugi-4CR reaction had been introduced to core structure and then made to react with other components to produce libraries of bio-active.

\textsuperscript{33} Azuaje, J.; El Maatougui, A.; Gareau-Mera, X.; Sotelo, E. ACS Comb. Sci. DOI:10.1021/co500036n.
compounds. Recently, our group have developed a method to synthesize optically active 1-benzylpyrrolidin-3-ol from natural product vasicine isolated from *Adhatoda vasica*.

1-Benzylpyrrolidin-3-ol is a privileged motif present in a broad range of naturally occurring alkaloids and biologically active molecules and commonly used as an intermediate for the preparation of a variety of drugs. Pharmaceuticals such as hypertensive bamidipine A, the quinolinone antibiotic clinafloxacin B, the muscarinic receptor antagonists darifenacine C, the carbapenem antibiotic RS-533, the anticoagulant DX-9065a, and the natural occurring detoxification agent detoxin A1-D2 comprise a pyrrolidinol subunit or a derivative thereof (Figure 6). However, the biological properties are highly dependent on the nature of the 1-benzylpyrrolidin-3-ol scaffold including its substituents, the propensity of hydrogen bond donor and acceptor and the electrostatic profile. Thus, keeping in view the importance of 1-benzylpyrrolidin-3-ol moiety in medicinal chemistry, it was envisaged to explore it for diversity-oriented synthesis of bioactive molecules as anticancer agents. We devised our objectives as follows:

2. *In vitro* screening of the synthesized analogues on human cancer cell lines.

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3.0. Results and discussion:

The primary amine functionality of 1-benzylpyrrolidin-3-ol (1) itself can be viewed as a potential amine component for the Ugi reaction with aldehydes, acids and isocyanides. However, the feasibility of proposed sequence relies heavily on the reactivity profile and space compatibility of the Ugi adducts. 1-Benzylpyrrolidin-3-ol (1), p-nitro benzaldehyde (2a), benzoic acid (3) and tert-buty1 isocyanide (4) were selected as model substrates. Equimolar amounts of these substrates were submitted to the standard U-4CR conditions in methanol at room temperature for 8 h. This process gave Ugi adducts (5a) satisfactorily in 90 % yields. The reaction was then subjected reaction with a set of aromatic aldehydes (2b-o), where the reaction behaviour did not seem to be sensitive to the increasing steric hindrance present in aldehyde variants and gave the corresponding products (5b-o) in good yields (54-78 %) (Scheme 1). Reaction possibly involves the formation of imine from 1-benzylpyrrolidin-3-ol (1) and corresponding aldehyde in the first step. In the next step benzoic acid (3) protonates the nitrogen atom of imine and thus making the substrate more available for nucleophilic attack. In the next step, the benzoic acid anion reacts with tert-buty1 isocyanide carbon (4) atom which further reacts with electrophilic carbon of imine in $S_N^2$ fashion. The isouamide intermediate thus formed undergoes intramolecular $N$-acyl transfer to give final stable 1a Ugi product (Figure 17).
Scheme 1. 1-Benzylpyrrolidin-3-ol derivatives involving different aldehydes groups in Ugi four component reaction.
Figure 17. Mechanism of the reaction.

The synthesized library of the compounds (5a-o) was screened for ability to induce cytotoxicity against panel of human cancer cell lines i.e., glioblastoma cell line (T98G), epidermoid carcinoma cell line (A431), lung carcinoma cell line (A549 & NCI-H322), using SRB assay and suspension human promyelocytic leukemia cell line (HL60) using MTT assay. The preliminary screening was done at 10 μM and 50 μM concentrations. All the compounds displayed a range of cytotoxicity towards these cancer cell lines, with the compound 5f bearing a trifluoromethoxy group at para-position of benzaldehyde presenting the maximum inhibition at 50 μM i.e. 100% cytotoxicity against glioblastoma cell line T98G, epidermoid carcinoma A431, lung carcinoma A549 and leukemia cell line HL60 and 95% cytotoxicity against lung carcinoma NCI-H322. At 10 μM conc., 5f was active against leukemia cell line HL-60 showing cytotoxicity of 52% (Table 1).
Table 1. Cytotoxicity data of the compounds (5a-o) synthesized by Ugi 4-CR.

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<th>Conc. (µM)</th>
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With 4-trifluoromethoxy benzaldehyde (5f) as a very potent residue for aldehyde component, we further investigated the structure activity relationship (SAR) with the variation in acid components. Using the synthesis protocol described above, we generated a follow-up library with 1-benzylpyrrolidin-3-ol (1), tert-butyl isocyanide (3), p-trifluoromethoxy benzaldehyde (4f) using a set of aromatic and aliphatic acids (2p-z) as substrates (Scheme 2). This process also resulted in good yields (38-72%) of Ugi adducts (5p-z), where the reactions conditions were tolerable to amino acid as well as monoprotected diacids.
Scheme 2 1-Benzylpyrrolidin-3-ol derivatives involving different acid groups in Ugi four component reaction.
The members of the synthesized library of the compounds (5p-z) was again screened for their ability to induce cytotoxicity against the above described panel of human cancer cell lines. Preliminary screening of the compounds was performed at 10 μM conc. where 5s and 5z were found to induce significant inhibition of cell growth in different panel of human cancer cell lines. The compound 5s showed the maximum growth inhibition against the cancer cell lines showing >50% cytotoxicity against epidermoid carcinoma A431, lung carcinoma NCI-H322, A549 and leukemia cell line HL-60 (Table 2). The analogue 5z showed >50% growth inhibition against leukemia cell line HL60 and >45% against epidermoid carcinoma A431 and lung carcinoma NCI-H322 and A549.

Table 2. Cytotoxicity data of the synthesised compounds.

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The analogues 5s and 5z were further evaluated at different concentrations to determine their IC₅₀ values on different cancer cell lines. The compound 5s exhibited IC₅₀ values of 7, 13, 7.3, 6.7, 2.5, 6, 8.7 and 12.9 μM in A431 (Epidermal), T98G (Glioblastoma), NCI-H322 (Lung), A549 (Lung), HL-60 (Leukemia), T47D (Breast), PC-3 (Prostate) and HCT-116 (Colon) cancer cell lines respectively. 5z also showed IC₅₀ values of 10.5, 15.8, 10.7, 10.5, 6.3, 10.5, 17.6 and 28 μM in A431, T98G, NCI-H322, A549, HL-60, T47D, PC-3 and HCT-116 cancer cell lines respectively. IC₅₀ values were determined by Graph Pad Prism5 software.
Table 3. IC₅₀ values of the compounds 5s and 5z.

<table>
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<th>Cpd</th>
<th>Conc. (μM)</th>
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<th>T98 G</th>
<th>A549</th>
<th>NCI-H322</th>
<th>HL-60</th>
<th>T47 D</th>
<th>PC-3</th>
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<td>6.3</td>
<td>10.0</td>
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</table>

![Graphical representation of IC₅₀ value of the compounds 5s (A) and 5z (B) on HL-60 cell lines.](image)

**Figure 18.** Graphical representation of IC₅₀ value of the compounds 5s (A) and 5z (B) on HL-60 cell lines.

To elucidate whether these compounds exert anticancer effect through apoptosis, further mechanistic work was carried out on HL-60 cells as 5s and 5z showed best anticancer activity in HL-60 leukemia cell line.

**Cell cycle analysis:** Regulation of cell cycle is a very important phenomenon in the life of a normal cell. Most of the differentiated cells are arrested in G1 phase but in case of cancer cells this control is lost and the cells go on dividing. The DNA content of the cell changes as the cell progresses through G1-S-G2 phases and this change can be detected by flow cytometry using fluorescent dye Propidium iodide (PI) that binds specifically to DNA. The treatment of human leukemia HL-60 cells with the compounds 5s and 5z exhibited a concentration dependent increase in hypo diploid sub-G₁ fraction indicative of apoptotic population. In 5s treated cells, sub-G₁ population increased with increasing conc. from 1.4%, 5% to 44.7% at 5 μM, 10 μM and 15 μM respectively, whereas untreated control showed 1.4% sub-G₁ DNA fraction. 5z also displayed a similar trend with 1.7%, 2.6% and 25.1% sub-G₁ population at 5 μM, 10 μM and 15 μM respectively.
Figure 19. Cell cycle analysis of 5s and 5z treated HL-60 cancer cells. HL-60 cells were treated with 5, 10 and 15 μM concentration of 5s and 5z for 24h to determine sub-G1 population indicative of DNA damage.

**Nuclear morphology of cells by fluorescence microscopy:** Nuclear morphological changes of cells were studied by fluorescence microscopy using DAPI staining. DAPI, a fluorescent probe specifically binds to the minor groove of A-T rich sequences of DNA and on binding its fluorescence is enhanced many fold. On treating the HL-60 cells with 5s and 5z at 5, 10 and 15 μM concentrations, characteristic changes of apoptosis were observed using DAPI staining and by analyzing under fluorescence microscopy. In contrast to normal cells, the apoptotic cells showed condensed and fragmented nuclear material. Untreated cells were spherical in shape, while the treated cells showed membrane blebbing, shrinkage and condensation of nuclear material. The results suggest that 5s and 5z induce apoptotic cell morphology in HL-60 cells.

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**Figure 20.** Fluorescence microscopy in HL-60 cells. The cells were treated with test compounds 5s and 5z at 5, 10 and 15 μM for 24h, stained with DAPI and observed to determine morphological changes. Untreated HL-60 cells showed normal nuclear morphology while positive control Camptothecin showed typical apoptotic bodies. Cells treated with test compounds 5s and 5z exhibited concentration dependent effect. The arrow in each case showed the appearance of apoptotic bodies.

**Effect on mitochondrial membrane potential loss:** The loss of mitochondrial membrane integrity is one of the early events leading to apoptosis. During apoptosis certain changes occur in mitochondria leading to loss of mitochondrial transmembrane electrical potential (ΔΨm) resulting in the release of cytochrome c into the cytoplasm which participates in the formation of apoptosome leading to the executioner phase of apoptosis. In order to determine whether 5s and 5z have any effect on mitochondrial membrane potential, RH-123 (6-amino-9-(2-methoxycarbonylphenyl)xanthen-3-ylidene]azanium chloride), a cationic fluorophore was used. In response to electric potential across the inner mitochondrial membrane, Rh-123 localises in the mitochondrial matrix, thus indicating that Rh-123 fluorescence is directly proportional to the mitochondrial membrane potential. HL-60 cells were treated with 5s and 5z to assess their effect on mitochondrial membrane potential loss. A concentration dependent loss of
mitochondrial membrane potential was observed in both the compounds. Untreated control displayed 3.1% loss whereas 4.2%, 6.9% and 72.8% loss was observed in case of 5s at 5, 10 and 15 μM concentrations respectively. 5z though not as potent as 5s, exhibited a similar trend of concentration dependent membrane potential loss displaying a loss of 3.8%, 5.9% and 18.4% at 5, 10 and 15 μM concentrations respectively.

**Figure 21.** HL-60 cells were incubated with 5, 10 and 15 μM concentrations of test compounds 5s and 5z for 24 h. Thereafter cells were stained with Rhodamine-123 (200 nM) for 1 h and analyzed by flow cytometry. Test compound 5s and 5z induced concentration dependent loss of mitochondrial membrane potential in HL-60 cells.

**Apoptosis induction by western blot analysis with PARP-1 and pro-caspase-3:** To confirm the induction of apoptosis by both compounds 5s and 5z, western blot analysis was performed for pro-caspase-3 and poly (ADP ribose) polymerase-1 (PARP-1). PARP-1 is activated and its level increases when DNA damage is induced in cells. As it repairs damaged DNA, its cleavage into 89kDa and 24kDa fragments by caspase-3 promotes apoptosis by preventing the DNA-repair induced survival signals.43 Caspases act as initiator and executioner of apoptotic cell destruction through a proteolytic cascade. Effector caspases on being activated by the initiator caspases are instrumental in dismantling the cell. Pro-caspase-3, an effector caspase, normally present as inactive

precursor in the cell on receiving apoptotic signal undergoes proteolytic cleavage to form active enzyme.\textsuperscript{44} The western blot analysis of PARP-1 cleavage and pro-caspase-3 expression in HL-60 cancer cell line was performed following 24h treatment with 5, 10 and 15 \( \mu \)M concentrations of \( 5s \) and \( 5z \). Densitometry of the proteins was done and normalized with \( \beta \)-actin for analysis. From the results it was observed that \( 5s \) and \( 5z \) induced cleavage of PARP-1 and pro-caspase-3, contributing towards activation of apoptotic pathways. Thus, these results along with cell cycle analysis, fluorescence microscopy and mitochondrial membrane potential analysis suggest that \( 5s \) and \( 5z \) exert anti-cancer effect by inducing apoptosis.

![Image](image.png)

**Figure 22.** A) Protein expression of PARP-1 and Pro-caspase-3 detected by western blot of 5, 10 and 15 \( \mu \)M concentration of \( 5s \) and \( 5z \) treated HL-60 cells. \( \beta \)-actin is shown as loading control; B) The graphs represent the densitometric scan of cleaved-Parp-1 and Pro-caspase-3 proteins, normalised by comparison with \( \beta \)-actin.

### 4.0. Conclusion:

In summary, we successfully applied Ugi 4-CR for diversity-oriented synthesis (DOS) in the generation of small libraries of structurally diverse compounds using 1-  

benzylpyrrolidin-3-ol as an amine source. The synthesized compounds were screened for
their anticancer activity, wherein the compound 5s and 5z displayed promising
antiproliferative activity, particularly in leukemia cells. The cell cycle analysis and
western-blot analysis with PARP-1 and procaspase-3 indicated that both 5s and 5z
induced apoptotic cell death in HL-60 cells. The promising antiproliferative and
apoptotic activity of 5s and 5z can be viewed as the lead for future drug discovery.

5.0. Experimental:

5.1. Cell Culture: Human epidermal cancer cell line A431, glioblastoma cell line
T98G, breast cancer cell line T47D, prostate cancer cell line PC-3, colon cancer cell line
HCT-116, leukemia cell line HL-60, lung cancer cell lines NCI-H322 and A-549 were
purchased from ECACC. Cells were maintained in RPMI/MEM/DMEM growth medium
supplemented with 10% FBS, 100 units/ml of penicillin and 100µg/ml of streptomycin.
Cells were grown in CO₂ incubator at 37° C temperature, 5% CO₂ and 95% humidity.

5.2. In Vitro cytotoxicity assay: The in vitro cytotoxicity of the compounds against
different adherent human cancer cell lines was determined by using SRB (sulforhodamine
B) assay.⁴⁵ Cells were seeded in 96 well tissue culture plates at a density of 8 x10⁵ to
15x10⁵ cells per 100µl/well and incubated at 37°C, 5% CO₂ and 95% relative humidity for
24 h. The test compounds (100µl/well) were added at indicated concentrations and
incubated for 48 h in CO₂ incubator. Cells were fixed with trichloroacetic acid 50%w/v
(after the incubation period is over) by gently layering on the top of the wells and plates
were incubated for 1h at 4 °C. The plates were thereafter washed with distilled water three
times and air dried. Cell growth was measured by adding SRB dye (0.4% w/v in 1% acetic
acid, 100 µl/well). The unbound dye was washed with 1% acetic acid 3 times and air dried.
The dye was dissolved in tris-buffer (100µl/well, 0.01M, pH 10.4) and plates were kept on
mechanical shaker for 10 min. The optical density (OD) was recorded at 540nm with
microplate reader (BioTek Synergy HT) and growth inhibition was calculated using the
following formula:

\[
\text{% Growth inhibition} = 100 - \left[ \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{test sample}} - \text{OD}_{\text{blank}}} \right] \times 100
\]

5.3. **In Vitro Proliferation Assay:** HL-60 cells were seeded in 96 well microtiter plates at a density of 15 x 10^3 cells/well and exposed to varying concentration of the test compounds for 48h. 20 μl of 2.5mg/ml of MTT dye was added to each well and incubated for 4 h before termination. Excess media was then blotted off and MTT purple formazan crystals were dissolved in 150 μl of DMSO. Optical Density was measured at 570nm with microplate reader (BioTek Synergy HT). IC₅₀ was determined by using Prism, version 5.04, from GraphPad Software (La Jolla, CA).

5.4. **Cell cycle analysis:** For cell cycle phase distribution analysis, HL-60 cells(5x10^5) were treated with varying concentrations of 5s and 5z compounds for 24 h, washed with PBS and fixed in ice cold 70% ethanol at -20°C, overnight. Cells were thereafter centrifuged and washed with PBS followed by the addition of RNase (100 μg/ml) at 37°C for 45 min and stained with propidium iodide to determine the cell cycle phase distribution. DNA fluorescence was measured using flow cytometer FACS Aria (Becton Dickinson, USA) and resulting DNA distributions were analyzed for the proportions of cells in apoptosis, G₁-phase, S-phase, and G₂-M phases of the cell cycle.⁴⁶

5.5. **Mitochondrial membrane potential:** To determine changes in mitochondrial membrane potential, Rhodamine-123 staining was done and analysed by flow cytometer. HL-60 cells (5x10⁵) were treated with different concentrations of 5s and 5z for 24hrs. Before termination of experiment, cells were treated with RH-123 (200nM) for 1 hr, centrifuged and washed with PBS. Cells were resuspended in PBS and fluorescence intensity was analyzed by BD-FACS Aria flow cytometer with an excitation wavelength of 488nm and emission wavelength of 525nm in FITC channel.⁴⁷

5.6. **Fluorescence microscopy:** To determine the nuclear morphological changes, HL-60 cells were stained with DNA specific fluorescent nuclear dye 4',6-diamidino-2-phenylindole (DAPI) and analysed under fluorescent microscope. Cells were treated with varying concentrations of 5s and 5z. After 24h incubation, cells were washed and resuspended in PBS. Smears of cells were made on glass slides, air dried, fixed in methanol for 20 min at -20 °C, again air dried and then stained in dark for 20 min with

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DAPI (1 μg/ml) at room temperature. Slides were then mounted using glycerol-PBS mixture (90:10) and analysed under fluorescence microscope (Olympus) using UV filter at 40X magnification.

5.7. Preparation of protein lysates, estimation and western blot analysis: After treatment with different concentrations of 5s and 5z, H1 60 cells (3x 10⁵) were harvested, washed with PBS and resuspended in lysis buffer containing RIPA and protease and phosphatase inhibitor cocktail. Cells were incubated on ice for 45 min with periodic vortexing and centrifuged at 14000xg for 15min. Supernatant was collected and stored at -20°C. The protein concentration was determined with QuantiPro BCA assay kit according to manufacturer’s protocol using Bovine Serum Albumin (BSA) as standard. An equal concentration of protein was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane at 4°C. The membrane was blocked with 3% BSA in TBS-Tween 20 and probed with primary PARP-1, Caspase-3 and β-actin and horseradish peroxidase linked respective secondary antibodies. The signals were detected by using western chemiluminescent HRP substrate and exposed to X-ray film for analysis.

5.8. Spectral data of synthesized compounds:

**Compound 5a:** Yield = 78%, ¹H NMR (500 MHz, CDCl₃) δ 8.34 – 8.18 (m, 2H), 8.12 – 7.98 (m, 2H), 7.72 – 7.65 (m, 2H), 7.49 – 7.40 (m, 4H), 7.33 (d, J = 7.0 Hz, 2H), 7.15 (s, 1H), 6.23 (d, J = 9.8 Hz, 1H), 5.15 (s, 1H, NH), 4.36 (t, J = 17.8, 22.7 Hz, 1H), 3.39 (m, 1H), 3.37 (dd, J = 19.1, 13.9 Hz, 1H), 2.83 (dd, J = 8.6, 5.3 Hz, 1H), 2.52 (m, 2H), 2.23 (m, 2H), 2.12 (m, 1H), 1.62 (m, 1H), 1.43-1.39 (m, 9H); ¹³C NMR (125 MHz, CDCl₃) δ 169.3, 169.8, 145.1, 142.9, 139.9, 135.5, 133.2, 131.0, 130.0, 129.5, 129.1, 128.5, 127.7, 127.5, 123.6, 71.9, 62.1, 61.3, 60.3, 53.9, 51.6, 37.5, 29.8; HR-ESI-MS (m/z) 531.2612 [M+H]⁺ (calcd for [C₁₀H₁₄N₂O₅+H]⁺ 531.2607).
**Compound 5b:** Yield = 64%, $^1$H NMR (500 MHz, CDCl$_3$) δ 7.99 (m, 1H), 7.85 – 7.73 (m, 2H), 7.68 (m, 1H), 7.64 (m, 1H), 7.42 (d, $J$ = 14.9 Hz, 2H), 7.45 – 7.33 (m, 4H), 7.28 (m, 1H), 7.17 (m, 1H), 6.12 (s, 1H), 5.35 (s, 1H, NH), 4.22 (m, 1H), 3.38 (m, 1H), 3.30 (m, 1H), 2.78 (dd, $J$ = 8.4, 5.2 Hz, 1H), 2.47 (d, $J$ = 10.8 Hz, 2H), 2.21 (m, 2H), 1.88 (m, 1H), 1.63 (m, 1H), 1.41 – 1.37 (m, 9H), 1.31 (s, 1H, OH). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.3, 169.8, 147.4, 142.8, 137.1, 135.3, 134.1, 133.1, 131.0, 130.3, 129.8, 129.5, 128.7, 128.5, 128.3, 127.7, 127.4, 121.5, 70.8, 62.6, 60.5, 60.1, 52.7, 51.7, 34.3, 29.7; HR-ESI-MS (m/z) 531.2648 [M+H]$^+$ (calkd for [C$_{30}$H$_{34}$N$_{4}$O$_{5}$+H]$^+$ 531.2607).

**Compound 5c:** Yield = 62%, $^1$H NMR (500 MHz, CDCl$_3$) δ 8.67 (s, 1H, NH), 7.82 – 7.74 (m, 2H), 7.57-7.47 (d, $J$ = 5.1 Hz, 2H), 7.43 – 7.35 (m, 2H), 7.34 (d, $J$ = 8.3 Hz, 2H), 7.32 – 7.28 (m, 2H), 7.23 (m, 1H), 7.10 – 7.04 (m, 2H), 6.01 (d, $J$ = 15.5 Hz, 1H), 4.37 (m, 1H), 3.39 (m, 1H), 3.35 (m, 1H), 3.09 (m, 1H), 2.71 (d, $J$ = 3.1 Hz, 2H), 2.46 (m, 2H), 1.88 (m, 1H), 1.64 (m, 1H), 1.36 – 1.32 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) 170.3, 169.8, 159.2, 139.9, 135.2, 133.1, 132.1, 131.9, 131.0, 130.8, 130.8, 130.0, 129.1, 128.5, 128.1, 127.7, 127.5, 116.1, 70.1, 63.6, 61.1, 60.2, 53.9, 52.4, 36.1, 29.6; HR-ESI-MS (m/z) 504.2668 [M+H]$^+$ (calkd for [C$_{30}$H$_{34}$FN$_{5}$O$_{5}$+H]$^+$ 504.2662).

**Compound 5d:** Yield = 63%, $^1$H NMR (500 MHz, CDCl$_3$) δ 7.98 – 7.81 (m, 2H), 7.64 (m, 1H), 7.51 (d, $J$ = 23.4 Hz, 2H), 7.42 (m, 4H), 7.38 – 7.32 (m, 3H), 7.19 (d, $J$ = 9.4 Hz, 2H), 5.98 (d, $J$ = 13.3 Hz, 1H, 1H), 4.14 (m, 1H), 3.52 (m, 1H), 3.26 (m, 1H), 2.71 (m, 1H), 2.65 (m, 2H), 2.46-2.23 (m, 2H), 2.16 (m, 1H), 1.88-1.79 (m, 1H), 1.62 (m, 1H), 1.40 – 1.36 (m, 9H), 1.29 (s, 1H, OH); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.3, 169.8, 142.6, 139.7, 139.2, 135.5, 133.2, 133.1, 132.5, 131.0, 130.0, 129.9, 129.1, 128.5, 127.9, 127.7, 127.5, 125.1, 70.8, 62.6, 62.1, 60.3, 52.9, 51.1, 34.4, 29.6; HR-ESI-MS (m/z) 546.1880 [M+H]$^+$ (calkd for [C$_{30}$H$_{34}$BrN$_{5}$O$_{5}$+H]$^+$ 546.1862).
**Compound 5e:** Yield = 60%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.91 - 7.80 (m, 2H), 7.63 - 7.41 (m, 5H), 7.31 (m, 3H), 7.20 (d, $J$ = 19.9 Hz, 2H), 7.03 (m, 1H), 6.24 (s, 1H, NH), 6.14 (m, 1H), 4.33 (m, 1H), 3.42 (m, 1H), 3.36 (m, 1H), 2.86 (m, 1H), 2.71 (m, 2H), 2.46 (d, $J$ = 1.8 Hz, 2H), 1.88 - 1.80 (m, 1H), 1.60 (m, 1H), 1.38 (m, 1H), 1.34 - 1.30 (m, 9H), $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 169.8, 138.6, 135.1, 134.3, 133.6, 133.1, 132.2, 131.0, 130.0, 129.7, 129.1, 128.6, 128.3, 127.1, 127.7, 126.3, 70.6, 62.6, 61.7, 59.8, 52.6, 50.5, 35.5, 29.3; HR-ESI-MS (m/z) 520.2371 [M+H]$^+$ (calcd for [C$_{30}$H$_{34}$ClN$_3$O$_5$]+ 520.2367).

**Compound 5f:** Yield = 68%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.90 - 7.76 (m, 2H), 7.49 (m, 1H), 7.45 - 7.33 (m, 5H), 7.31 - 7.21 (m, 2H), 7.19 (m, 1H), 7.05 - 6.91 (m, 2H), 5.97 (m, 1H), 4.51 (s, 1H, NH), 4.33 (m, 1H), 3.87 (m, 1H), 3.75 (m, 1H), 2.69 (d, $J$ = 22.4 Hz, 2H), 2.64 (m, 1H), 2.46 (m, 2H), 2.13 (m, 1H), 1.88 (m, 1H), 1.62 (m, 1H), 1.50 (m, 1H), 1.33 - 1.29 (m, 9H), $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 169.2, 168.7, 150.5, 139.6, 135.5, 134.5, 133.2, 132.2, 131.0, 130.0, 129.1, 128.5, 127.7, 127.5, 125.0, 123.2, 123.1, 120.8, 70.5, 62.6, 61.3, 60.7, 52.5, 50.8, 34.3, 29.7; HR-ESI-MS (m/z) 570.2601 [M+H]$^+$ (calcd for [C$_{31}$H$_{34}$F$_3$N$_3$O$_4$]+ 570.2580).

**Compound 5g:** Yield = 63%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.99 - 7.86 (m, 2H), 7.50 (m, 1H), 7.48 - 7.30 (m, 7H), 7.30 (m, 1H), 7.16 (m, 1H), 6.98 (m, 1H), 6.03 (s, 1H), 5.38 (s, 1H, NH), 4.29 (m, 1H), 3.82 (m, 1H), 3.30 (m, 1H), 2.97 (m, 1H), 2.71 (m, 2H), 2.47 (d, $J$ = 7.8 Hz, 2H), 1.89 (m, 1H), 1.64 (m, 1H), 1.31 - 1.27 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 169.8, 163.0, 160.9, 139.9, 136.1, 136.1, 135.5, 133.2, 131.0, 130.11, 130.0, 129.2, 129.2, 129.1, 128.5, 127.7, 127.5, 119.0, 118.8, 110.8, 110.6, 70.4, 62.6, 60.2, 52.7, 50.8, 35.3, 29.6; HR-ESI-MS (m/z) 582.1754 [M+H]$^+$ (calcd for [C$_{30}$H$_{33}$BrF$_3$N$_3$O$_4$]+ 582.1768).
**Compound 5h:** Yield = 58%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.96 (m, 1H), 7.89 – 7.74 (m, 3H), 7.71 (m, 1H), 7.53 (m, 1H), 7.48 – 7.30 (m, 7H), 7.30 (m, 1H), 7.16 (d, $J = 9.4$ Hz, 2H), 6.13 (s, 1H), 4.51 (s, 1H, NH), 4.38 (m, 1H), 3.54 (m, 1H), 3.46 (m, 1H), 2.79 (m, 1H), 2.71 (m, 2H), 2.46 (m, 2H), 2.12 (m, 1H), 1.88 (m, 1H), 1.62 (m, 1H), 1.35 – 1.31 (s, 9H), 1.26 (s, 1H, OH); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 169.8, 139.2, 136.5, 135.5, 135.2, 134.1, 133.2, 131.1, 130.3, 129.7, 129.1, 128.7, 128.5, 128.5, 128.4, 127.7, 127.5, 126.9, 126.9, 126.4, 69.9, 62.6, 60.3, 52.3, 50.4, 34.7, 29.8; HR-ESI-MS (m/z) 536.2941 [M+H]$^+$ (calcd for [C$_{34}$H$_{37}$N$_3$O$_5$]+ 536.2913).

**Compound 5i:** Yield = 60%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.88 – 7.79 (m, 5H), 7.47 (m, 1H), 7.39 (m, 4H), 7.32 (m, 1H), 7.19 (m, 2H), 5.97 (d, $J = 4.5$ Hz, 1H), 4.46 (m, 1H), 3.81 (m, 1H), 3.65 (m, 1H), 2.93 (m, 1H), 2.71 (m, 2H), 2.46 (m, 2H), 2.37 (m, 1H), 1.87 (m, 1H), 1.63 (m, 1H), 1.36 – 1.32 (m, 9H), 1.20 (s, 1H, OH); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 169.5, 168.8, 147.4, 145.3, 139.9, 137.4, 135.3, 135.2, 135.0, 134.9, 134.6, 133.6, 131.0, 129.1, 128.5, 127.4, 125.3, 123.0, 122.9, 121.6, 70.6, 62.6, 60.9, 52.9, 35.5, 29.8; HR-ESI-MS (m/z) 622.5592 [M+H]$^+$ (calcd for [C$_{32}$H$_{35}$F$_6$N$_3$O$_5$]+ 622.2504).

**Compound 5j:** Yield = 68%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.98 – 7.86 (m, 2H), 7.78 (d, $J = 4.5$ Hz, 1H), 7.53 (m, 1H), 7.48 (m, 1H), 7.45 – 7.38 (m, 3H), 7.31 (t, $J = 7.3$, 2.4 Hz, 1H), 7.31 – 7.20 (m, 3H), 7.14 (m, 1H), 6.01 (m, 1H), 4.52 (m, 1H), 3.97 (m, 1H), 3.43 (m, 1H), 2.96 (m, 1H), 2.71 (m, 2H), 2.46 (m, 2H), 1.89 (m, 1H), 1.64 (m, 1H), 1.42 (m, 1H), 1.38 – 1.34 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 162.3, 151.0, 147.0, 140.4, 139.9, 135.5, 133.2, 131.0, 130.0, 129.1, 128.5, 127.7, 127.6, 127.5, 121.9, 93.4, 71.9, 62.6, 61.8, 60.3, 53.9, 51.6, 37.5, 29.9; HR-ESI-MS (m/z) 487.2713 [M+H]$^+$ (calcd for [C$_{28}$H$_{34}$N$_4$O$_5$]+ 487.2709).
**Compound 5k:** Yield = 72%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.66 - 8.52 (m, 8H), 8.03 - 7.91 (m, 8H), 7.50 (s, 4H), 7.48 - 7.42 (m, 8H), 7.38 (dd, $J = 28.5$, 0.6 Hz, 17H), 7.31 (t, $J = 2.7$ Hz, 1H), 7.30 (s, 4H), 7.16 (s, 4H), 6.04 (s, 4H), 5.82 (s, 4H), 4.36 (s, 3H), 3.81 (s, 4H), 3.30 (s, 4H), 2.98 (s, 3H), 2.71 (s, 4H), 2.45 (d, $J = 11.1$ Hz, 7H), 1.89 (s, 4H), 1.64 (s, 3H), 1.40 - 1.32 (m, 39H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 169.8, 150.3, 139.9, 138.5, 137.2, 135.8, 135.5, 133.4, 133.2, 131.0, 130.0, 129.1, 128.5, 127.7, 127.5, 123.5, 71.1, 62.6, 60.3, 53.9, 51.3, 35.5, 29.6; HR-ESI-MS (m/z) 487.2719 [M+H]$^+$ (calcd for [C$_2$H$_{34}$N$_4$O$_3$+H]$^+$ 487.2709).

**Compound 5l:** Yield = 75%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.02 (m, 1H), 7.83 (m, 1H), 7.83 - 7.71 (m, 2H), 7.53 (d, $J = 12.6$ Hz, 1H), 7.47 (m, 2H), 7.42 (m, 2H), 7.39 - 7.20 (m, 5H), 7.14 (m, 1H), 6.12 (s, 1H), 5.28 (s, 1H, NH), 4.34 (m, 1H), 3.58 (m, 1H), 3.38 (m, 1H), 2.86 (m, 1H), 2.68 (d, $J = 28.9$ Hz, 2H), 2.46 (m, 1H), 1.89 (m, 1H), 1.64 (m, 1H), 1.32 - 1.28 (s, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 169.8, 169.2, 151.7, 145.5, 139.9, 136.7, 136.1, 135.5, 133.2, 131.8, 131.5, 131.0, 130.1, 130.0, 129.1, 128.3, 127.8, 127.7, 126.5, 122.6, 119.9, 70.5, 62.6, 60.3, 52.2, 51.1, 33.5, 29.5; HR-ESI-MS (m/z) 537.2853 [M+H]$^+$ (calcd for [C$_3$H$_{36}$N$_4$O$_3$+H]$^+$ 537.2866).

**Compound 5mc:** Yield = 64%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.12 (s, 1H), 8.11 (m, 1H), 7.93 - 7.85 (m, 3H), 7.62 (m, 1H), 7.53 (d, $J = 9.8$ Hz, 2H), 7.45 - 7.34 (m, 5H), 7.25 (d, $J = 5.8$ Hz, 2H), 7.07 (m, 1H), 5.95 (s, 1H), 4.42 (m, 1H), 3.78 (m, 1H), 3.22 (m, 1H), 2.93 (m, 1H), 2.71 (m, 2H), 2.48 (d, $J = 15.4$ Hz, 2H), 1.89 (m, 1H), 1.64 (m, 1H), 1.34 - 1.28 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 166.3, 151.4, 143.2, 139.7, 137.6, 137.1, 135.5, 133.7, 133.2, 131.0, 130.0, 129.7, 129.5, 128.5, 127.7, 127.5, 126.8, 124.9, 124.7, 70.6, 62.7, 60.3, 53.8, 51.1, 35.4, 29.3; HR-ESI-MS (m/z) 537.2860 [M+H]$^+$ (calcd for [C$_3$H$_{36}$N$_4$O$_3$+H]$^+$ 537.2866).
**Compound 5n**: Yield = 58%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.79 (s, 1H), 7.93 – 7.81 (m, 2H), 7.52 (m, 1H), 7.44 (m, 1H), 7.42 – 7.36 (m, 2H), 7.35 – 7.20 (m, 3H), 7.14 (m, 1H), 7.04 (m, 1H), 6.87 (m, 1H), 5.82 (s, 1H), 4.52 (m, 1H), 3.98 (d, $J$ = 23.0 Hz, 1H), 3.27 (m, 1H), 2.93 (m, 1H), 2.71 (m, 2H), 2.44 (d, $J$ = 25.6 Hz, 2H), 2.23 (m, 1H), 1.89 (m, 1H), 1.63 (m, 1H), 1.35 – 1.31 (m, 9H), 1.27 (brs, 1H, OH); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.1, 166.8, 139.9, 136.3, 135.5, 133.2, 132.5, 131.0, 130.0, 129.1, 128.5, 128.4, 127.7, 127.6, 127.2, 125.6, 70.7, 62.5, 60.3, 53.9, 51.6, 34.7, 29.7; HR-ESI-MS (m/z) 492.2354 [M+H]$^+$ (calcd for [C$_{28}$H$_{33}$N$_3$O$_3$S+H]$^+$ 492.2321).

**Compound 5o**: Yield = 54%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.98 – 7.86 (m, 2H), 7.48 (m, 1H), 7.46 – 7.40 (m, 2H), 7.33 (d, $J$ = 1.3 Hz, 2H), 7.14 (m, 1H), 6.26 (m, 1H), 4.48 (m, 1H), 3.91 (m, 1H), 3.28 (m, 1H), 3.15 (m, 1H), 2.71 (m, 2H), 2.46 (d, $J$ = 1.1 Hz, 2H), 1.89 (m, 1H), 1.64 (m, 1H), 1.35 – 1.31 (m, 9H), 1.23 (brs, 1H, NH); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 168.9, 150.7, 148.8, 145.5, 144.4, 143.4, 139.9, 135.5, 133.2, 131.0, 130.3, 129.1, 128.5, 127.7, 127.5, 70.4, 63.5, 62.6, 60.3, 53.2, 51.0, 35.4, 29.8; HR-ESI-MS (m/z) 576.2298 [M+H]$^+$ (calcd for [C$_{30}$H$_{31}$F$_3$N$_3$O$_3$+H]$^+$ 576.2286).

**Compound 5p**: Yield = 56%, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.40 (m, 1H), 7.67 – 7.53 (m, 2H), 7.48 – 7.26 (m, 2H), 7.26 – 7.17 (m, 1H), 7.17 – 7.09 (m, 1H), 7.09 – 6.92 (m, 2H), 6.25 (m, 1H), 5.34 – 5.26 (m, 1H, NH), 4.33 – 4.11 (m, 1H), 3.39 (t, $J$ = 14.8 Hz, 1H), 3.29 – 3.07 (m, 1H), 2.65 – 2.54 (m, 1H), 2.54 – 2.37 (m, 2H), 2.36 – 2.28 (m, 1H), 2.22 – 1.96 (m, 2H), 1.73 – 1.59 (m, 1H), 1.51 – 1.22 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 168.5, 167.4, 150.5, 149.5, 136.5, 136.1, 135.9, 132.6, 132.2, 131.7, 131.4, 130.8, 130.6, 128.6, 128.2, 127.7, 122.3, 121.1, 120.5, 70.9, 66.9, 62.5, 55.2, 52.4, 51.7, 34.7, 28.5; HR-ESI-MS (m/z) 571.2548 [M+H]$^+$ (calcd for [C$_{30}$H$_{31}$F$_3$N$_3$O$_4$+H]$^+$ 571.2532).
**Compound 5q**: Yield = 54%, $^1$H NMR (500 MHz, CDCl$_3$) δ 8.84 (d, $J = 22.4$ Hz, 1H), 8.38 (m, 1H), 8.22 (dd, $J = 22.4$, 16.4 Hz, 1H), 7.48 – 7.32 (m, 1H), 7.32 – 7.24 (m, 2H), 7.22 – 6.99 (m, 2H), 6.20 – 6.09 (m, 1H), 4.34 – 4.16 (m, 1H), 3.64 – 3.45 (m, 1H), 3.45 – 3.25 (m, 1H), 2.83 (m, 2H), 2.78 (m, 1H), 2.67 (m, 2H), 2.57 (m, 2H), 2.39 (m, 2H), 2.24 (m, 2H), 2.05 (m, 1H), 1.66 (m, 1H), 1.43 – 1.30 (m, 9H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 167.8, 167.2, 149.4, 149.2, 145.0, 144.7, 142.6, 137.1, 132.4, 132.1, 131.5, 130.7, 130.0, 129.8, 128.6, 127.6, 121.0, 120.6, 120.5, 77.1, 71.0, 67.2, 63.0, 55.4, 53.2, 49.1, 46.4, 44.1, 51.8, 34.7, 28.5; HR-ESI-MS (m/z) 578.2962 [M+H]$^+$ (calcd for [C$_{29}$H$_{38}$F$_3$N$_2$O$_4$+H]$^+$ 578.2954).

**Compound 5r**: Yield = 70%, $^1$H NMR (500 MHz, CDCl$_3$) $^1$H NMR (400 MHz, CDCl$_3$) δ 8.62 – 8.40 (m, 1H), 7.88 (t, $J = 11.9$ Hz, 1H), 7.68 – 7.48 (m, 2H), 7.43 – 7.27 (m, 3H), 7.27 – 7.17 (m, 2H), 7.16 – 7.09 (m, 1H), 7.02 (dd, $J = 23.8$, 7.9 Hz, 1H), 6.21 (s, 1H), 6.04 – 5.91 (m, 1H, NH), 4.29 – 4.20 (m, 1H), 3.47 – 3.30 (m, 1H), 3.28 – 3.17 (m, 1H), 2.68 – 2.53 (m, 1H), 2.53 – 2.29 (m, 2H), 2.27 – 2.04 (m, 2H), 1.73 – 1.50 (m, 2H), 1.35 (d, $J = 9.7$ Hz, 9H), 1.34 – 1.32 (brs, 1H, OH); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 167.7, 167.2, 151.5, 149.4, 141.2, 138.7, 136.6, 134.3, 134.3, 133.09, 132.5, 131.5, 130.9, 128.8, 128.4, 127.9, 124.1, 121.5, 120.3, 119.5, 116.4, 77.0, 70.9, 66.9, 62.3, 55.2, 52.5, 34.8, 29.6; HR-ESI-MS (m/z) 649.1689 [M+H]$^+$ (calcd for [C$_{30}$H$_{35}$BrF$_2$N$_2$O$_4$+H]$^+$ 649.1637).

**Compound 5s**: Yield = 62%, $^1$H NMR (400 MHz, CDCl$_3$) δ 8.48 (dd, $J = 9.5$, 5.2, Hz, 2H), 7.97 – 7.75 (m, 1H), 7.67 – 7.47 (m, 1H), 7.43 – 7.29 (m, 2H), 7.29 – 7.12 (m, 3H), 7.11 – 6.98 (m, 2H), 6.16 (s, 1H), 4.35 – 4.26 (m, 1H), 3.49 – 3.37 (m, 1H), 3.33 – 3.21 (m, 1H), 2.78 – 2.64 (m, 1H), 2.61 – 2.46 (m, 1H), 2.35 (dd, $J = 15.5$, 7.0 Hz, 1H), 2.22 – 1.97 (m, 2H), 1.79 – 1.55 (m, 1H), 1.39 – 1.34 (m, 9H), 1.26 (s, 2H); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 167.7, 167.2, 151.5, 149.4, 147.5, 147.4, 138.7, 138.1, 137.5, 132.1, 131.0, 130.4, 130.7, 129.1, 128.1, 121.3,
120.7, 71.1, 67.2, 62.6, 55.2, 52.5, 34.8, 29.7; HR-ESI-MS (m/z) 682.1297 [M+H]+ (calcd for [C_{31}H_{32}BrClF_{3}N_{3}O_{4}+H]^+ 682.1295).

**Compound 5t**: Yield = 72%, $^1$H NMR (500 MHz, CDCl$_3$) δ 7.94 - 7.79 (m, 2H), 7.48 - 7.36 (m, 3H), 7.36 - 7.22 (m, 3H), 7.17 (d, J = 17.0 Hz, 2H), 7.05 - 6.90 (m, 2H), 6.03 (m, 1H), 4.39 (m, 1H), 3.45 (m, 1H), 3.38 (m, 1H), 2.99 (m, 1H), 2.71 (m, 2H), 2.48 (d, J = 16.5 Hz, 2H), 1.89 (m, 1H), 1.64 (m, 1H), 1.37 - 1.33 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 169.7, 168.9, 155.9, 139.9, 136.3, 134.5, 134.5, 132.2, 130.0, 129.1, 128.8, 128.7, 127.7, 127.5, 125.0, 123.2, 123.2, 123.1, 122.9, 120.8, 118.7, 70.5, 62.6, 60.3, 55.7, 53.5, 51.3, 36.5, 29.8; HR-ESI-MS (m/z) 604.2202 [M+H]+ (calcd for [C_{31}H_{33}ClF_{3}N_{3}O_{4}+H]^+ 604.2190).

**Compound 5u**: Yield = 68%, $^1$H NMR (500 MHz, CDCl$_3$) δ 7.89 (m, 1H), 7.45 - 7.36 (m, 2H), 7.33 (m, 2H), 7.27 (t, J = 7.8, 2.9 Hz, 2H), 7.02 - 6.89 (m, 1H), 6.13 (s, 1H), 4.79 (m, 1H), 4.37 (m, 1H), 3.51 (m, 1H), 3.39 (m, 1H), 2.96 (m, 1H), 2.71 (m, 1H), 2.44 (d, J = 24.7 Hz, 2H), 2.39 (m, 1H), 1.89 (s, 1H), 1.64 (s, 1H), 1.43-139 (s, 9H), 1.35 (d, J = 12.5 Hz, 3H), 1.33 - 1.29 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.5, 169.0, 158.0, 150.9, 140.0, 134.5, 133.8, 132.2, 130.7, 129.2, 127.3, 125.0, 123.1, 122.9, 120.8, 118.7, 80.6, 71.9, 62.6, 60.3, 55.7, 51.6, 50.5, 37.5, 29.8, 27.9, 18.5; HR-ESI-MS (m/z) 637.3276 [M+H]+ (calcd for [C_{32}H_{34}F_{3}N_{3}O_{6}+H]^+ 637.3213).

**Compound 5v**: Yield = 64%, $^1$H NMR (500 MHz, CDCl$_3$) δ 7.44 - 7.36 (m, 2H), 7.34-7.22 (m, 4H), 6.99 - 6.90 (m, 2H), 6.13 (m, 1H), 4.52 (m, 1H), 4.48 (m, 1H), 4.39 (m, 1H), 4.23 (m, 1H), 3.54 (m, 1H), 3.41 (m, 1H), 2.91 (m, 1H), 2.76 (m, 1H), 2.71 (m, 1H), 2.53 (m, 1H), 2.46 (m, 1H), 2.27 (m, 1H), 1.89 (m, 1H), 1.62 (m, 1H), 1.45 - 1.41 (m, 9H); $^{13}$C NMR (125
MHz, CDCl$_3$) δ 169.8, 169.7, 156.3, 150.8, 140.0, 134.5, 133.8, 132.3, 132.3, 130.5, 129.2, 127.8, 127.3, 123.2, 123.2, 121.8, 79.3, 70.8, 62.2, 60.4, 54.5, 53.9, 51.6, 34.6, 29.5, 27.1; HR-ESI-MS (m/z) 653.3169 [M+H]$^+$ (calcd for [C$_3$H$_3$F$_3$N$_4$O$_6$]+ 653.3162).

**Compound 5w:** Yield = 38%, $^1$H NMR (500 MHz, CDCl$_3$) δ 8.89 (s, 1H, NH), 8.38 – 8.24 (m, 2H), 8.23 – 8.08 (m, 2H), 7.48 – 7.33 (m, 3H), 7.33 – 7.28 (m, 2H), 7.16 (m, 1H), 7.05 – 6.91 (m, 2H), 6.13 (s, 1H), 4.45 (m, 1H), 3.41 (m, 1H), 3.00 (m, 1H), 2.71 (m, 2H), 2.65 (m, 1H), 2.46 (m, 1H), 2.30 (m, 1H), 1.86 (m, 1H), 1.62 (m, 1H). 1.39 – 1.35 (m, 9H), 1.24 (brs, 1H, OH); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 169.5, 168.8, 150.9, 149.1, 139.9, 134.5, 133.2, 132.2, 130.0, 129.1, 129.0, 127.7, 127.5, 125.0, 124.0, 123.2, 123.1, 122.9, 120.8, 118.7, 70.5, 62.6, 61.3, 56.8, 53.9, 51.6, 36.3, 29.8; HR-ESI-MS (m/z) 615.2457 [M+H]$^+$ (calcd for [C$_{31}$H$_{33}$F$_3$N$_4$O$_6$]+ 615.2430).

**Compound 5x:** Yield = 55%, $^1$H NMR (500 MHz, CDCl$_3$) δ 7.43 – 7.32 (m, 3H), 7.29 (m, 1H), 7.25 (m, 1H), 7.02 – 6.87 (m, 3H), 6.14 (m, 1H), 4.26 4.56 (m, 1H), (m, 1H), 3.57 (m, 1H), 3.28 (m, 1H), 2.91 (m, 1H), 2.79 (m, 1H), 2.71 (m, 1H), 2.40 (t, $J = 23.7$ Hz, 2H), 2.37 – 2.31 (m, 3H), 2.20 (s, 1H), 1.88 (s, 1H), 1.82 – 1.66 (m, 4H), 1.63 (m, 1H), 1.36 – 1.32 (m, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 174.6, 171.1, 169.8, 150.8, 140.1, 134.5, 134.4, 132.3, 132.3, 131.1, 129.3, 127.9, 127.1, 123.2, 123.2, 121.8, 70.8, 62.6, 62.0, 60.4, 55.3, 50.2, 51.6, 37.6, 35.6, 33.8, 29.7, 24.1, 23.2; HR-ESI-MS (m/z) 608.2949 [M+H]$^+$ (calcd for [C$_{31}$H$_{40}$F$_3$N$_4$O$_6$]+ 608.2947).

**Compound 5y:** Yield = 52%, $^1$H NMR (500 MHz, CDCl$_3$) δ 7.45 – 7.31 (m, 3H), 7.29 (d, $J = 1.2$ Hz, 2H), 7.24 (m, 1H), 7.07 – 6.88 (m, 3H), 6.14 (m, 1H), 4.47 (m, 1H), 3.75 (s, 3H), 3.39 (m, 1H), 3.01 (m, 1H), 2.87 (m, 1H), 2.71 (m, 2H), 2.36 (dd, $J = 38.4, 22.8$ Hz, 3H), 2.32 – 2.27 (m, 3H), 1.88 (m, 1H), 1.78 – 1.69 (m, 4H), 1.64 (m, 1H), 1.54-151 (m, 3H). 3.7 – 1.30 (m, 9H); $^{13}$C
NMR (125 MHz, CDCl₃) δ 174.6, 171.1, 169.8, 150.8, 139.6, 134.5, 134.4, 132.3, 132.3, 131.1, 129.3, 127.9, 127.1, 123.2, 123.2, 121.8, 70.7, 62.6, 62.0, 60.4, 53.9, 51.8, 51.6, 37.6, 35.4, 33.8, 29.7, 28.9, 25.5, 23.1; HR-ESI-MS (m/z) 636.3290 [M+H]⁺ (calcd for [C₃H₄F₃N₂O₆+H]⁺ 636.3260).

**Compound 5z:** Yield = 52%,¹H NMR (500 MHz, CDCl₃) δ 7.38 (m, 1H), 7.31 (d, J = 3.3 Hz, 2H), 7.24 – 7.17 (m, 2H), 7.11 (m, 1H), 6.99 – 6.91 (m, 2H), 6.24 (s, 1H), 5.21 (s, 1H, NH), 4.30 (m, 1H), 3.45 (m, 1H), 3.34 (m, 1H), 2.81 (m, 1H), 2.71 (m, 1H), 2.61 2.46 (m, 2H), 2.23 (d, J = 9.8 Hz, 2H), 2.16 – 2.03 (m, 2H), 1.89 (d, J = 4.5 Hz, 2H), 1.84 – 1.78 (m, 6H), 1.63 (m, 1H), 1.40 (m, 1H), 1.34 – 1.25 (m, 9H), 1.03 (bs, 1H, OH), 0.98 – 0.91 (m, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 170.0, 169.8, 150.8, 140.1, 134.5, 131.2, 131.1, 129.8, 129.3, 124.4, 124.1, 123.1, 120.8, 119.7, 71.9, 62.6, 60.3, 55.5, 53.9, 51.6, 41.9, 37.5, 36.2, 32.9, 29.6, 26.3, 25.2, 20.1, 17.9; HR-ESI-MS (m/z) 618.3524 [M+H]⁺ (calcd for [C₃H₄F₃N₂O₆+H]⁺ 618.3519).
NMR spectras:

$^1$H and $^{13}$C NMR spectra of 1-benzylpyrrolidin-3-ol:
$^1$H and $^{13}$C NMR spectra of compound 5p:
$^1$H and $^{13}$C NMR spectra of compound 5r:
$^1$H and $^{13}$C NMR spectra of compound 5z.
$^1$H and $^{13}$C NMR spectra of compound 5z:
Summary

The thesis entitled “Isolation, Synthesis of Bioactive Molecules and Development of New Synthetic Methodologies” consists of three chapters.

Chapter 1: Introduction

Chapter 2: Isolation and Bio-evaluation of secondary metabolites from microbes

Chapter 3: Vinyl acetate mediated cross-aldol reactions of acetaldehyde

Chapter 4: Diversity-oriented synthesis using 1-benzylpyrrolidin-3-ol

Chapter 1: Introduction

This chapter gives an idea of organization of the thesis alongwith the general introduction to the importance of the areas chosen for work.

Chapter 2: Isolation and Bio-evaluation of secondary metabolites from microbes

Terrestrial and marine microorganisms are abundant source of structurally diverse bioactive substances and have provided important contributions to the discovery of bioactive molecules. A large number of anticancer drugs like anthracyclines, mitosanes, bleomycins, epothilones etc. have also been isolated from microbes; even the most used anticancer agent taxol can also be produced from endophytic fungi Taxomyces andreanae and Nodulisporium sylviforme. The immense potential that microbes offer for developing new therapeutic agents can also be inferred from the fact that of all the known natural products from natural sources, only 20-25% is biologically active, out of which 10% are of microbial origin, though they account for only 5% of all the natural products known.

One of the prime objectives of the present work has been isolation and identification of diverse range of bioactive molecules including exploring the possibilities of identifying new scaffolds from the microbial sources particularly from the microbial repository of the institute comprising bacterial and fungal endophytes. The extraction and isolation strategy would include protocols analogous to that proposed by NCI, USA, followed by their structural elucidation and bio-evaluation of the anticancer potential.

We have selected endophytes from

i) Endophytic fungi associated with Cedrus deodara.

ii) Endophytic fungi associated with Grewia asitatica.

iii) Soil bacteria associated with Bacillus subtilis.
Summary

After extraction, all the extracts were subjected to extensive chromatographic separation, which resulted in the isolation and purification of several interesting molecules characterized through NMR spectroscopy and establishment of configuration in some cases by X-ray crystallography. The complete structural elucidation of as well as assignment of all $^1$H and $^{13}$C NMR signals was also based on 2D NMR experiments (HMBC, HSQC and COESY).

(i) Endophytic fungi associated with Cedrus deodara:

From an endophytic fungus, a close relative of Talaromyces sp., found in association with Cedrus deodara, four compounds were isolated and characterized. Two isolates i.e. 7-hydroxy ramulosin (3) and (1S,3R,5R)-3-methyl-2-oxacyclo[3.3.1]nonan-7-one (4) were new to the literature. The structures of two compounds (1 and 4) were confirmed by X-ray crystallography.

![Figure 1. Structures of the isolated compounds 1-4 from the endophyte of Cedrus deodara](image)

The compounds displayed a range of cytotoxicities against human cancer cell lines (HCT-116, A-549, HEP-1, THP-1, and PC-3). All the compounds were found to induce apoptosis in HL-60 cells, as evidenced by fluorescence and scanning electron microscopy studies. Also, the compounds caused significant microtubule inhibition in HL-60 cells (Table 1).

Table 1. Cytotoxicity of the isolated molecules

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conc. (µM)</th>
<th>Lung A-549</th>
<th>Liver HEP-1</th>
<th>Leukemia THP-1</th>
<th>Leukemia</th>
<th>Prostate PC-3</th>
<th>Prostate</th>
<th>Colon HCT-116</th>
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<td>59</td>
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</table>
**Summary**

**Scanning electron microscopy.** In an attempt to decipher the insights of cancer specific cell death, the compound 1-4 were evaluated for their ability to induce apoptosis. In the present study we used scanning electron microscope (SEM), as the morphological analysis by electron microscopy constitutes a very important and unambiguous method to identify the specific type of cell death. For SEM, HL-60 cells both in control and treatment groups were processed, and it revealed that HL-60 cells in control were spherical in shape having microvilli on entire surface and with a few surface projections (Figure 4A-B). The compounds 1-4 respectively on incubation of the cells at 30 μM for 30h caused condensation, smoothening of cell surface and blebbing of plasma membrane in few cells which represents the morphological features of apoptotic cell (data not shown). However at 50 μM, after 30 h of treatment with isolated compounds, the number of cells and features of apoptosis increased in all the treated cells, to various degrees (Figure 2 C-H).

![Figure 2. A-H, Scanning electron microscopy of untreated (A, B), 2 (C, D) and 3 (E, F) and 4 (G, H) treated HL-60 cells showing surface ultrastructure. The untreated cells show microvilli on cell surface (A, B, arrow). The treatment of 50 μM of 2 (C, D) and 3 (E, F) and 4 (G, H) respectively after 30 h causes condensation, smoothening of cell surface and blebbing of plasma membrane (C, D, E, F, G, H white arrow) and formation of apoptotic bodies (G, H asterisk). (Magnification A, C, E, G 2000X; B, D, F, G 5000X).](image-url)
**Summary**

**Fluorescence electron microscopy.** The results were further corroborated by the studies for nuclear morphological changes of HL-60 cells, carried out to gain insight into the morphological alterations caused by these compounds. The HL-60 cells were chosen since most of isolated molecules were found to be active against this leukemia cell line. The HL-60 cells were treated with 50 µM of either compound for 30h and subsequently stained with DAPI. Cells were observed under fluorescence microscope (40x). The untreated cells have large sized intact nuclei (Figure 3A). The treatment at 50 µM induced the condensation and fragmentation of nuclei (arrow) due to apoptosis (Figure 3 B-E) representing respectively four isolated compounds. The more number of apoptotic cells were seen in treatment with compound 2.

![Image](image1.png)

**Figure. 3.** Effect of compound 1 to 4 on nuclear morphology of HL-60 cells. The cells were treated with 50 µM of different compound for 30 h and subsequently stained with DAPI as described in Materials and methods. Cells were observed under fluorescence microscope (40x). The untreated cells (A) have large sized intact nuclei. All the four compounds, 1(B), 2(C), 3(D) and 4(E) induced the condensation and fragmentation of nuclei (arrow) due to of apoptosis. The more number of apoptotic cells were seen in treatment with compound 2 (D).

**Immunofluorescence microscopic studies.** Microtubules are attractive targets for the chemotherapeutic agents. Since these play important roles in the regulation of the mitotic progression, disrupting the assembly of microtubules can induce cell cycle arrest in M.
phase and trigger the signals for apoptosis. Interestingly, the microtubule inhibitors, such as vinca alkaloids, colchicinoids and combretastatin inhibit tubulin polymerization and the microtubule promoters, such as taxanes and epothilones, promote or stabilize the

![Figure 4](image)

**Figure 4.** Effect of compounds 1 to 4 on microtubules in THP-1 cells. Cells were plated on cover slips. After 24 h, the cells were treated with 1-4 for 48 h, at 50 μM for 48 h. Immunocytochemical staining was conducted using anti-α-tubulin antibody and Alexa Flour-488-labeled secondary antibody (right). Nuclei were stained with DAPI (left). The data is representative of three separate set of experiments.

tubulin polymer form. In addition it is well accepted that the microtubule inhibitors can induce apoptosis caused by cell cycle arrest. In this connection we investigated the effect of isolated compounds 1-4 on the microtubules in leukemia cells so as to evaluate the possible target for the isolated compounds in the present study. We determined the effect of compounds 1-4 on microtubule structure using confocal microscopy. Figure 4 shows confocal images of microtubule structures at 48 h of treatment. At the 50 μM, cells treated
with 1-4 showed a remarkable disruption and loss of microtubules compared to the control. Paclitaxel treatment at 1μM for 24 h caused the stabilization of tubulin polymer form.

(ii) Endophytic fungi associated with *Grewia asiatica*:

Seven molecules have been isolated from the above endophytes and their structures have been elucidated from $^1$H and $^{13}$C NMR experiments and comparing with reported literature.

![Structures of isolated compounds](image)

*Figure 5. Structures of the isolated compounds 5-11 from endophyte of Grewia Asiatica.*

The major concern associated with microbial natural products is their synthetic production which recently has been addressed by moving beyond the traditional methods of growing micro-organism under normal laboratory conditions. As we know that micro-organism consists of enzyme coded biosynthetic gene clusters that regulates the production of secondary metabolites.

To test the effect of epigenetic modifiers on *A. fumigatus*, 100 μM of commercially available HDAC inhibitors (suberoylanilide hydroxamic acid (SAHA), sodium butyrate (NaB), valproic acid (VA)) and DNA-methyltransferase inhibitors (5-azacytidine) was added to the different cultures during the inoculation. Comparative profiling with LC-ESI-MS and TLC demonstrate that cultures treated with SAHA, NaB and 5-azacytidine did not produce new or enhanced secondary metabolite profile while a significant change was observed in VA treated fungal culture. LC-ESI-MS spectra of VA treated fungal culture showed the enhancement in the production of secondary metabolites as well as a intense
peak at retention time 10.9 min with \( m/z \) of 444.1 [M+H]+ which was present in traces in control. TLC profiling also showed the presence of a UV visible spot which wasn’t visible in control culture at \( \text{rf} \) 0.70 in 5 % MeOH:DCM.

![Structure](image)

**Figure 5.** Structures of isolated compound 12

**Quantification of isolated secondary metabolites in extract.** For quantification, 1 mg of extract was dissolved in 1 ml of MeOH and subjected to LC-ESI-MS analysis. The LC-ESI-MS analysis of MeOH fraction in total ion current (TIC) mode showed seven major peaks with mass \( m/z \) 454.1 (at \( t_R \) 4.6 min), 454.2, (at \( t_R \) 6.4 min), \( m/z \) 417.1 (at \( t_R \) 7.5 min), 444.1 (at \( t_R \) 10.9 min), \( m/z \) 352.4 (at \( t_R \) 11.3 min), \( m/z \) 349.3 (at \( t_R \) 12.7 min), \( m/z \) 359.1 (at \( t_R \) 13.6 min) and 458.2 (at \( t_R \) 18.9 min) as depicted in Figure 6. These peaks were identified as pseurotin F2 (6), pseurotin A (5), Pseurotin D (7), fumiquinazoline C (12), tryprostatin C.

**Table 2.** Quantified content of isolated compounds in extract through LC-ESI-MS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Extract (1mg) (( \mu g ))</th>
<th>Extract having VA(1mg) (( \mu g ))</th>
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<td>26.716</td>
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<td>11</td>
<td>34.517</td>
<td>33.034</td>
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<tr>
<td>12</td>
<td>4.612</td>
<td>49.300</td>
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*Quantification of 6 was not done due to same mass ion peak.*
(8), gliotoxin (10), bis(methylthio)gliotoxin (11), and fumagillin (9) after running a LC-MS chromatogram of extract sample with LC-ESI-MS chromatogram of pure reference standards through spiking experiment.

**Table 3.** Cytotoxicity data of isolated compounds against panel of cancer cell lines.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Conc. (µM)</th>
<th>Lung A-549</th>
<th>Prostate PC-3</th>
<th>Breast MCF-7</th>
<th>Leukemia HL-60</th>
<th>Pancreatic MIA-PaCa-2</th>
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</table>

The isolated molecules displayed a range of cytotoxicity from good to moderate as shown in Table 3.

**(iii) Soil bacteria associated with Bacillus subtilis:**

Four molecules have been isolated from the above endophytes and their structures elucidated on the basis of 1D and 2D NMR spectroscopy and comparing with reported literature.

![Figure 6. Structures of isolated the compounds 13-16 from Bacillus Subtilis.](image-url)
Summary

Table 4. Cytotoxicity data of isolated compounds against panel of cancer cell lines.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Conc. (µM)</th>
<th>Lung A-549</th>
<th>Prostate PC-3</th>
<th>Breast MCF-7</th>
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Chapter 3: Vinyl acetate mediated cross-aldol reactions of acetaldehyde

In the context to the development of new methods, one of the key transformations in organic chemistry is C-C bond formation which acts as a backbone in the synthesis of plethora of molecules. The importance of C-C bond formation can be inferred from the wide research that has gone into the reactions like aldol, Diels-Alder, Michael, allylic alkylation, cross coupling metathesis etc.

In the recent past there have been many advances in development of organocatalysts with each allowing greater scope in terms of coupling partners (nucleophiles and electrophiles) and milder approaches. Various nucleophiles have made their way into the world of chemical diversity through these synthetic methodologies. Despite all the advances the simplest of all nucleophiles, acetaldehyde was not much explored due to the following reasons: a) Its low boiling point (21 ºC); b) Its ability to act as a nucleophile as well as electrophile gives rise to uncontrolled cascade reactions like polyaldolization, double Mannich reaction etc; c) Its tendency to polymerize at room temperature; sometimes freshly distilled acetaldehyde is required for the product formation in good yields; d) Dehydration of the final product enables Michael type additions; e) Its high reactivity results in self-aldol condensation prior to cross-coupling; f) Undergo Tishchenko-type processes; g) The small steric difference between the methyl and hydrogen atom suffers with low relative control, resulting in decreased enantioselectivity.
Summary

Based on these challenges and complications, we envisaged using vinyl acetate, which is cheap and commercially available, as a precursor of acetaldehyde for the following chemical transformations:

A. Vinyl acetate mediated cross aldol reaction of acetaldehyde.

B. Synthesis of α,β-unsaturated δ lactones

As it is well established that vinyl/isopropenyl acetate in the presence of lipase undergoes hydrolysis with the release of acetaldehyde/acetone therefore, we designed employing lipase catalyzed \textit{in situ} generation of active form of acetaldehyde and acetone from vinyl acetate and isopropenyl acetate respectively, followed by organocatalyzed aldol reaction with aromatic aldehydes for the preparation of β-hydroxy aldehyde/ketones.

\textbf{Table 1.} The effect of various lipases, bases and solvents on aldol reaction of vinyl acetate and p-nitro benzaldehyde.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent$[^{a}]$</th>
<th>Lipase</th>
<th>Base</th>
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<th>Yield$[^{b}]$</th>
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