Chapter 2

Synthesis and biological applications of 1,3-benzoxazines and pyrido-1,4-oxazin-3-ones

Chapter 2a: 1,3-Benzoxazines

2.1. Introduction

Diabetes mellitus, particularly its subtype 2 (T2-DM), is considered to be a major and increasing threat to human health and accounts for an estimated more than 300 million cases of diabetes worldwide [1,2]. Diabetes is associated with a significant number of comorbidities, such as cardiovascular disorders, stroke, diabetic retinopathy and kidney dysfunction, and long-term complications may even lead to limb amputations, among other consequences [2].

In addition to injectable insulin and analogs thereof, four distinct classes of oral hypoglycemic agents are currently used for the treatment of T2-DM. In addition to metformin as an oral drug used for the early control of T2-DM, there are a number of second and third-line pharmacological agents available, such as sulfonylureas, thiazolidinediones, incretin-based remedies, and α-glucosidase inhibitors. Given the increased perception that handling the early stages of diabetes is of crucial importance, several recent studies and approaches are focusing on agents that can delay or inhibit glucose absorption. Delaying glucose absorption, such as by blocking glycoside hydrolases (particularly α-glucosidases), allows extended time for β-cells to increase insulin secretion, and thereby reduce circulatory glucose levels [3,4].

α-glucosidases are membrane-bound enzymes that catalyze the selective hydrolysis of glycosidic bonds in oligosaccharides, polysaccharides, and their conjugates to release glucose and the respective mono saccharides. Although they occur throughout living organisms, most of them are
located in the brush border of the small intestine to facilitate glucose uptake [4,5]. Thus, the use of α-glucosidase inhibitors (AGIs) for the treatment of T2-DM delays the release of glucose and halts glucose absorption, thereby lowering the postprandial blood glucose level and improving prediabetic conditions. The currently most prominent AGIs are acarbose (Glucobay), a natural compound from an Actinoplanes strain, and the N-hydroxyethyl analogue of 1-deoxynojirimycin, miglitol [4,6]. However, these compounds have been reported to cause severe gastrointestinal complications.

Numerous efforts have been made to further develop AGIs in order to improve treatment of prediabetic states [7,8]. Glycosides, the natural substrates for the glycoside hydrolases which are composed of an aglycone and a glycan moiety, have recently attracted particular attention in this field. Stable β-glucosides such as 2-(2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one)-β-D-glucopyranoside and desmethoxy derivatives including 6,7-dimethoxy-benzoazolin-2(3H)-one, 4-hydroxy-2H-1,4-benzoazin-3(4H)-one, and 4-acetylbenzoazolin-2(3H)-one, are found in living plants [9]. These glucosides are biologically inactive, but enzymatically converted to active aglycones such as DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one) and the desmethoxy derivative DIBOA, by β-glucosidases [10]. These products have diverse effects, that include antiauxin, antiinflammatory, and powerful antibiotic activities. These aglycones are further degraded spontaneously to the corresponding benzoazolinones, MBOA (6-methoxy-benzoazolin-2(3H)-one) and the desmethoxy derivative ROA. Degradation is faster if the benzene ring and amide linkage bears electron donating or hydroxyl groups [11], and these chemical variations are what we explored in more detail in the current work. In addition to this, our aim was to remove the relatively labile N-hydroxy amide moiety via replacement by a suitable ring system, masking the glycosylation site of glycoside (Scheme 29). These efforts
have the goal of increasing efficacy of the compounds in vivo, in order to lower blood sugar levels by inhibiting glycosidase.

Scheme 29: Proposed strategy for the synthesis of 1,3-benzoxazines

2.2. Results and Discussion

2.2.1. Chemistry

2.2.1.1. Chloro acetic acid mediated synthesis of 1,3-benzoxazines from amino-benzyl alcohols and aldehydes

This work is based upon the previous synthesis of an oxazine derivative which was able to mimic the pyranoside structure of glycans functionally [12]. In our current research, a series of novel 1,3-benzoxazines 3 (a-n) (Table 1) bearing different substituents were synthesized using 2-aminobenzyl alcohols and different aldehydes in chloroacetic acid via aerobic oxidative synthesis as shown in Scheme 30.

Scheme 30: Schematic representation for the synthesis of 1,3-benzoxazines
Table 1: Structures and yields of newly synthesized 1,3-benzoxazine based aglycones

<table>
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<th>Compound</th>
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<th>$R_2$</th>
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<th>Yield ($%$)</th>
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<td>H</td>
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</tr>
<tr>
<td>3f</td>
<td>H</td>
<td><img src="image" alt="Structure" /></td>
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<td>----</td>
<td>------</td>
<td>----------------------------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
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<td>Cl</td>
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<td>83</td>
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<tr>
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<tr>
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<td><img src="image" alt="Chemical Structure" /></td>
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<tr>
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<tr>
<td>3n</td>
<td>H</td>
<td><img src="image" alt="Chemical Structure" /></td>
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<td>91</td>
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</tbody>
</table>

*yield calculated after column purification*

2.2.2. Biological studies

The above design principles led to the synthesis of 14 compounds (Table 1) whose α-glucosidase inhibitory activity was validated both in vivo as well as in vitro, and supported by computational approaches as described below. The synthesized aglycones inhibited both α-glucosidase and α-amylase activity, with overall relatively similar IC₅₀ values between 11 μM and 60 μM (Table 2), and compound 3g exhibiting strong inhibition of both glucosidases, with respective IC₅₀ values of 11 μM and 11.5 μM. The addition of phenolic and halogen substituents to the 1,3-benzoxazine ring was found to increase inhibitory potency, whilst the incorporation of

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a flavone moiety decreases potency (compounds 3c, 3j, and 3m). 1,3-benzoxazines bearing an electron withdrawing chlorine substituent were found to be more potent against α-glucosidase, whereas the electron donating methyl group was not particularly favoured. Introduction of an imidazole ring (compound 3f), to give 1,3-benzoxazine, resulted in an enhanced inhibition (IC$_{50}$=16 μM), while a chromene moiety decreased activity of the compound 3m.

**Table 2:** Inhibitory efficacy of benzoxazine library against α-glucosidases

<table>
<thead>
<tr>
<th>Entry</th>
<th>α-Glucosidase IC$_{50}$ (μM)</th>
<th>α-Amylase IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>17.1±0.1</td>
<td>20.4±0.2</td>
</tr>
<tr>
<td>3b</td>
<td>32.0±0.2</td>
<td>59.2±1.0</td>
</tr>
<tr>
<td>3c</td>
<td>21.6±0.1</td>
<td>10.6±0.3</td>
</tr>
<tr>
<td>3d</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>3e</td>
<td>NS</td>
<td>23.3±0.5</td>
</tr>
<tr>
<td>3f</td>
<td>16.7±1.0</td>
<td>18.5±0.1</td>
</tr>
<tr>
<td>3g</td>
<td>11.5±0.1</td>
<td>11.0±0.3</td>
</tr>
<tr>
<td>3h</td>
<td>27.7±1.0</td>
<td>26.4±0.5</td>
</tr>
<tr>
<td>3i</td>
<td>27.8±0.2</td>
<td>22.2±0.1</td>
</tr>
<tr>
<td>3j</td>
<td>31.9±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>3k</td>
<td>23.8±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>3l</td>
<td>20.5±0.5</td>
<td>17.6±0.3</td>
</tr>
<tr>
<td>3m</td>
<td>NS</td>
<td>51.0±0.3</td>
</tr>
<tr>
<td>Acarbose</td>
<td>4.3±0.04</td>
<td>4.4±0.02</td>
</tr>
</tbody>
</table>

- NS - not significant

To study the efficacy of the potent α-glucosidase inhibitor, compound 3g (4-(7-chloro-2,4-dihydro-1H-benzo[d][1,3]oxazin-2-yl)phenol), on glucose uptake *in vivo* maltose and
sucrose tolerance test were carried out on overnight fasted experimental rats by taking acarbose as a positive control as well as glucose uptake by porcine diaphragm by using insulin as a enhancer. It can be seen that acarbose (at 3mg/kg) significantly reduced the plasma glucose concentration at 30, 60, 90 min time intervals in starved rats treated with maltose as substrate compared to maltose control (Figure 1A). In sucrose fed rats differences are less pronounced and only significant at 60 and 90 min time points (Figure 1B). At a concentration of 50 mg/kg body weight, compound 3g inhibited glucose uptake in rats fed with maltose, which was similar to acarbose treatment at 30 and 60 min. However, compound 3g significantly reduced plasma glucose concentration at the 90 min time point compared to acarbose, indicating a different Pharmacokinetic/Pharmacodynamic (PK/PD) profile of compound 3g on between these compounds. Furthermore, compound 3g significantly reduced the plasma glucose concentration throughout all time points (0-180 min) compared to the acarbose treated group when sucrose was used as a substrate (Figure 1B). At a concentration of 100 mg/kg bodyweight, compound 3g was significantly more effective than the acarbose standard at all time points. In order to establish possible synergistic effects between compound 3g and acarbose, plasma glucose levels were measured in starved rats fed individually with maltose and sucrose at different time points up to 180 minutes. In both cases, synergistic activity of compound 3g (50 mg/kg bodyweight) and acarbose (3 mg/kg bodyweight) prevented substrate utilization, and the plasma glucose concentration remained unchanged when compared to the saline treated group.
Figure 1: *In vivo* maltose and sucrose oral uptake inhibition by compound 3g. *In vivo* effect of compound 3g and acarbose on plasma glucose concentration by oral (A) maltose and (B) sucrose tolerance test. Values are presented as mean ± SEM (n=5). *p<0.05, **p<0.01, ***p<0.001 significant compared to respective maltose/sucrose alone treated groups.

The inhibitory efficacy of compound 3g on rat intestinal glucosidases (maltase and sucrase) was also evaluated. The compound showed both maltase and sucrase inhibitory activities in a dose-dependent manner (Figures 2A and 2B). Acarbose inhibited both the intestinal glucosidases activity with an IC₅₀ of 3.5 µM (maltase) and 4 µM (sucrase), while compound 3g was found to be two-fold selective for maltase (IC₅₀=10 µM) over sucrase (IC₅₀=20 µM). Furthermore, acarbose and compound 3g synergistically inhibited intestinal maltase more efficiently compared to intestinal sucrase (Figure 2C and 2D).
Figure 2: *In vivo* intestinal sucrase and maltase inhibition by **compound 3g** and acarbose. Percentage inhibition of compound 3g on intestinal maltase (A) and sucrase (B). Percentage inhibition of intestinal maltase (C) and sucrase (D) induced by the compound 3g in presence of acarbose. Percentage inhibition is presented as mean 6 SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 significant compared to acarbose.

**Compound 3g** is six-fold selective for maltase over sucrase (IC_{50}=10 μM and 60 μM, respectively) and shows clean dose-response relationships both *in vitro* as well as *in vivo*.

Further, porcine diaphragm was used in order to understand the effect of **compound 3g** on insulin mediated glucose uptake via GLUT4. The glucose transporter isoforms GLUT4 and GLUT1 are highly expressed in muscle cells, with GLUT4 being more abundant in an intracellular compartment from which it is quickly translocated to the plasma membrane as a response to insulin challenge. Both insulin-dependent and noninsulin-dependent diabetes were shown to reduce glucose utilization in muscle either due to a defective expression or
dysregulation in GLUT4 translocation [3, 13]. In the present study, glucose uptake was found to
be normal in the control group treated with no insulin. However as expected, significant
increased glucose uptake was observed in diaphragm treated with insulin (1U) compared to
control. We found that compound 3g at a dose of 50 and 100 µM significantly blocked the
glucose uptake both in control and insulin treated diaphragm in a dose-dependent manner
(Figure 3).

Figure 3: In vitro glucose transport across the porcine membrane in the presence and absence of
insulin and compound 3g. Values are presented as mean ± SEM of three independent
experiments. ***p<0.001 significant compared to insulin alone treated diaphragm.
2.2.3. In silico target prediction

In addition to experimental validation of the intended target α-glucosidase shown above, in silico target prediction [14,15] was performed with the full set of 14 synthesized compounds, in order to obtain a more comprehensive impression of the bioactivity profile of the synthesized 1,3-benzoxazine derivatives. It was found that only Compound 3g, the most active in the series, is predicted to target the Sodium/glucose co transporters 1 and 2, which may contribute to the in vivo efficacy of this compound. However, no experimental validation of this additional target has been performed.

2.2.4. Molecular Docking studies:

In order to hypothesize a binding mode, molecular docking has been performed between compound 3g and maltase-glucoamylase (Figure 4). The crystal structure of MGAM-C (Human maltase-glucoamylase C terminal domain) in complex with its inhibitor Acarbose (PDB ID: 3TOP) was used as a model to determine the molecular interaction between enzyme and the synthesized 1,3-benzoxazine derivatives [16]. In MGAM, both the N-and C-terminal domains (MGAM-N and MGAM-C) carry out the same catalytic function with different substrate specificities. The MGAM-C hydrolyzes linear α-1,4-linked oligosaccharide substrates and plays a pivotal role in the production of glucose in the human lumen and considered as an efficient drug target for T2-DM. Since, there is no information regarding the co-crystal structure of murine glucosidase and acarbose, we have used the co-crystal structure of MGAM-C and acarbose for docking studies. The synthesized 1,3-benzoxazine derivatives have relatively similar IC_{50} values, Dock Score (column ‘DS’) in particular seems to show higher scores for the more active compounds (3g, 3a and 3f) as opposed to less active compounds (3b, 3h, 3i and 3j) (Table 3). Structurally, the most active compound 3g, binds deep in the MGAM catalytic
domain (Figure 5), in which the chlorobenzisoxazine ring stacks into the hydrophobic cluster of Tyr1251, Trp1355, Trp1369, Tyr1427, Phe1559, and Phe1560, along with the phenolic ring is stacked to Tyr1251, His1584, Trp1418, and Trp1523. The terminal hydroxyl group of the phenolic component of compound 3g shows hydrogen bonding with Asp1279 and Ile1280, which are also involved in hydrogen bonding with the terminal hydroxyl group of Acarbose in the co crystal. In addition, the exposed oxygen atom in the benzoxazine ring of compound 3g appears to show ionic interaction with Asp1526 and Arg1510, which is also crucial in the Acarbose-MGAM co crystal. These results clearly suggest that both acarbose and compound 3g shares similar binding pattern towards MGAM-C.

Figure 4: Interaction map of MGAM catalytic domain co-crystallized with acarbose. The labelled key amino acids are represented as a stick model with the carbon atom as green, and other atoms in their parent colours. The binding of acarbose, whose carbon atom is coloured in
pink and other atoms with their parent colour. The hydrogen bonding is represented as dark dotted line.

Table 3: Computational analysis of the binding of aglycones towards α-amylase:

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<thead>
<tr>
<th>Compounds</th>
<th>MW</th>
<th>LS1D</th>
<th>LS2D</th>
<th>PLP1</th>
<th>PLP2</th>
<th>JAIN</th>
<th>-PMF</th>
<th>-LE</th>
<th>DS</th>
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<td>56.46</td>
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<td>2.1</td>
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<td>3b</td>
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<td>-9.4</td>
<td>64.3</td>
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</table>

LS1D and LS2D: LigScore1D and LigScore2D are a fast, simple, scoring function for predicting receptor-ligand binding affinities. PLP1 and PLP2: Piecewise Linear Potentials are fast, simple, docking function that has been shown to correlate well with protein-ligand binding affinities. JAIN: An empirical scoring function (lipophilic interactions, polar attractive interactions, polar repulsive interactions, solvation of the protein and ligand, and an entropy term for the ligand) through an evaluation of the structures and binding affinities of a series of protein-ligand complexes. PMF: potential of mean force is the scoring function developed based on statistical analysis of the 3D structures of protein-ligand complexes. LE: Ligand internal energy, the internal non bonded ligand energy is calculated for each new conformation that is generated. DS: Dock Score: Candidate ligand poses are evaluated and prioritized according to the Dock Score function.
Figure 5: Interaction map of MGAM catalytic domain co-crystallized with Compound 3g. The labelled key amino acids are represented as a stick model with carbon atom as green, and other atoms with their parent colour. The binding of compound 3g, whose carbon atom is coloured in pink and other atoms with their parent colour. The hydrogen bonding is represented in dark dotted line.

2.2.5. Chem-informatics based Target prediction for Compound 3g

Finally, we applied a metabolite prediction software, namely MetaPrint2D-React[17], to bioactive compound 3g and found the most likely metabolic site to be a glucuronidation site with a (significant) normalised occurrence ratio of less than 0.33 and but greater than 0.15 (Figure 6). Hence, this study for the first time demonstrated the design, synthesis, and
characterization of novel 1,3-benzoxazine aglycones and their validation \textit{in vitro} and \textit{in vivo}. \textbf{Compound 3g} significantly inhibited rat intestinal glucosidases, namely maltase and sucrase, in a dose-dependent fashion and led to decreased blood sugar levels in starved rat model. In addition to this, \textbf{compound 3g} acts synergistically with Acarbose in lowering the blood sugar levels to that of the saline control alone.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{compound_3g.png}
\caption{Proposed mechanism of action of \textbf{compound 3g} on glucosidases and it’s predicted in silico glucuronidase sites.}
\end{figure}

\textbf{2.3. Conclusion:}

This study demonstrated the novel synthesis of benzoxazine glycones and their effective inhibition towards glucosidases. The newly synthesized compounds showed better IC\textsubscript{50} values for both \(\alpha\)-glucosidase and \(\alpha\)-amylase, ranging from 11-60 \(\mu\)M, and are found effective when
compared to natural substrate aglycones, such as BOA and derivatives that deteriorate faster in aqueous solution. The *in silico* molecular docking studies revealed that benzoxazines bind to the catalytic domain of MGAM, correlating with a high DS for the most active compound 3g. The docking score of compound 3g and binding poses were found to be similar with the anti-diabetic drug acarbose. Furthermore, studies of *in silico* target prediction algorithms showed that compound 3g potentially targets the sodium-glucose co transporter 1. Both *in vitro* and *in vivo* experimental results suggested an anti-hyperglycemic effect of compound 3g, which significantly inhibits glucose uptake in starved rat model by blocking intestinal maltase and sucrase. Evidently, compound 3g was determined to possess the glucuronidase site, which potentially converts it into a stable glycoside *in vivo*. The aglycones synthesized in this study could hence constitute a novel pharmacological starting point for the treatment or alleviation of T2DM and its secondary complications. However, further studies elucidating interaction between compound 3g and specific glucose transporters would be highly exciting.

2.4. Experimental section:

2.4.1. Chemistry

The progress of all reactions was monitored by TLC, which was performed on 2.0-5.0 cm aluminum sheets precoated with silica gel 60 F 254 to a thickness of 0.25 mm (Merck) using UV light for visualization. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker/Agilent NMR spectrometer operating at 400 and 100 MHz, respectively. Chemical shifts are given in $\delta$ values (ppm) using tetramethylsilane as the internal standard. Mass spectra were recorded using liquid chromatography mass spectrometry (LCMS). Infrared spectra were recorded in KBr on
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Shimadzu FT-IR model 8300 spectrophotometer. Chromatographic separations were carried out on 60:120 silica gel.

All solvents and reagents were purchased from standard firms (Sigma Aldrich, Merck) and which were used as such without further purification. Chromatographic purification was conducted by column chromatography over 120 mesh silica gel using hexane-EtOAc as eluent.

2.4.1.1. General procedure for the preparation of N-substituted indole and imidazole aldehydes:

2.4.1.1.1. 4’-((3-formyl-1H-indol-1-yl)methyl)-[1,1’-biphenyl]-2-carbonitrile

This compound was obtained from indole-3-carbaldehyde (1 eq.), 4’-(bromomethyl)-[1,1’-biphenyl]-2-carbonitrile (1.2 eq.), potassium carbonate (2.5 eq.), and DMF (8 mL) as a solvent and stirring for 14 h at RT. Melting point 121-122 ℃; \(^1\)H NMR (DMSO-d\(_6\), 400 MHz): δ 9.73 (s, 1H, aldehyde-II), 8.99 (d, J=6.2 Hz, 1H, Ar-II), 8.85-7.36 (m, 12H, Ar-II), 5.61 (s, 2H); Mass: ESI-MS 337.13(M+1)^+. Elemental analysis calculated for C\(_{23}\)H\(_{16}\)N\(_2\)O: C, 82.12; H, 4.79, N, 8.33; found C, 79.85; H, 4.98; N, 8.52 %.

2.4.1.1.2. 2-butyl-4-chloro-1-(4-nitrobenzyl)-1H-imidazole-5-carbaldehyde

This compound was prepared by using reported protocol (18), briefly, 2-butyl-4-chloro-1H-imidazole-5-carbaldehyde (1 eq.), 1-(bromomethyl)-4-nitrobenzene (1.2 eq.), potassium carbonate (2.5 eq.), and DMF (8 mL) as a solvent and stirring for 14 h at RT. Melting point 86-87 ℃.
2.4.1.2. General Procedure for the synthesis of benzoxazines: To a 50 mL round bottom flask, amino benzyl alcohol (1.0 eq.), aldehyde (1.0 equiv), chloro acetic acid (1.0 eq.) and methanol (10 mL) were added. The reaction mixture was stirred at room temperature for 16 h., the reaction was monitored by TLC, after the completion of the reaction the methanol was evaporated and then water was added and extracted with ethyl acetate. The organic layer was dried by using anhydrous sodium sulphate, and concentrated in vacuum to afford compound benzoxazine as a crystalline solid. The compounds were then purified by column chromatography using hexane-EtOAc as eluent.

2.4.1.3. Characterization data of isolated 1,3-Benzoxazine compounds

4-(2,4-dihydro-1H-benzo[d][1,3]oxazin-2-yl)phenol (3a):

This compound was obtained as brownish solid in 83% yield. Melting point: 160-162 °C; \(^1\text{H NMR}\) (CDCl\(_3\), 400 MHz): \(\delta\) 8.06-6.70 (m, 8H, Ar-H), 5.28 (s, 1H, -CH), 5.02 (s, 1H, O-H), 4.90-4.80 (m, 2H, CH\(_2\)), 4.13 (s, 1H, N-H); \(^1\text{C NMR}\) (CDCl\(_3\), 100 MHz): \(\delta\) 70.05, 97.95, 114.64, 120.47, 126.08, 126.13, 129.13, 129.79, 130.22, 138.07, 147.90, 155.51.

Elemental analysis calculated for C\(_{14}\)H\(_{13}\)NO\(_2\): C, 73.99; H, 5.77; N, 6.16 %; found C, 73.68; H, 5.49; N, 5.98 %; MASS: m/z found for C\(_{14}\)H\(_{13}\)NO\(_2\) was 228.2 ([M+1]).

2-(1H-indol-3-yl)-2,4-dihydro-1H-benzo[d][1,3]oxazine (3b):

This compound was obtained as black color solid in 82% yield. Melting point: 65-67 °C; \(^1\text{H NMR}\) (CDCl\(_3\), 400 MHz): \(\delta\) 11.80 (s, 1H, indole-NH), 8.64-8.01 (m, 4H, Ar-H), 7.50-7.07 (m, 5H, Ar-H), 5.12 (s, 1H, -CH), 4.70 (s, 2H, -CH\(_2\)), 4.25 (s, 1H, N-H); Elemental analysis calculated for C\(_{16}\)H\(_{14}\)N\(_2\)O: C, 76.78 %; H, 5.64 % ; N, 11.19 %; found C, 76.54 %; H,
5.51 %; N, 11.53 %; MASS: m/z found for C_{10}H_{14}ClN_{2}O was 251.3 ([M+1]^+).

2-(2-methyl-1H-indol-3-yl)-2,4-dihydro-1H-benzo[d][1,3]oxazine(3c):
This compound was obtained as black color solid in 87% yield. Melting point: 70-72 °C; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz): \(\delta\) 10.12 (s, 1H, indole N-H), 8.22-8.06 (m, 5H, Ar-H), 7.26 (m, 3H, Ar-H), 5.19 (s, 1H, -CH\textsubscript{2}-), 4.83 (s, 2H, -CH\textsubscript{2}-), 4.13 (s, 1H, -N-H), 2.11 (s, 3H, -CH\textsubscript{3}); Elemental analysis calculated for C\textsubscript{17}H\textsubscript{16}N\textsubscript{2}O; C, 77.25 %; H, 6.10 %; N, 10.60 %; found C, 77.14 %; H, 6.11 %; N, 10.43 %; MASS: m/z found for C\textsubscript{17}H\textsubscript{16}N\textsubscript{2}O was 264.4 ([M+1]^+).

4'-(3-(2,4-dihydro-1H-benzo[d][1,3]oxazin-2-yl)-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carbonitrile (3d):
This compound was obtained as brown color solid in 86% yield. Melting point: 122-124 °C; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz): \(\delta\) 8.35-7.26 (m, 17H, Ar-H), 5.61 (s, 2H, Ar-CH\textsubscript{2}), 5.42 (s, 1H, -CH), 4.7 (s, 2H, -CH\textsubscript{2}), 4.09 (s, 1H, -NH); Elemental analysis calculated for C\textsubscript{30}H\textsubscript{23}N\textsubscript{3}O; C, 81.61 %; H, 5.25 %; N, 9.52 %; found C, 81.14 %; H, 5.11 %; N, 9.43 %. MASS: m/z found for C\textsubscript{30}H\textsubscript{23}N\textsubscript{3}O was 442.4 ([M+1]^+).

3-(2,4-dihydro-1H-benzo[d][1,3]oxazin-2-yl)-4H-chromen-4-one (3e):
This compound was obtained as brownish solid in 89% yield. Melting point: 142-144 °C; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz): \(\delta\) 8.55 (s, 1H, Ar-O-CH), 8.32-7.07 (m, 8H, Ar-H), 5.32 (s, 2H, -CH\textsubscript{2}), 4.80 (s, 1H, -CH), 4.11 (s, 1H, NH); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 100 MHz): \(\delta\) 70.1, 85.5, 110.6, 115.2, 116.1, 119.6, 122.0, 127.6, 127.8, 129.2, 132.6, 133.6, 142.2, 149.1, 155.6,
179.2. Elemental analysis calculated for C_{17}H_{12}NO_3: C, 73.11%; H, 4.69%; N, 5.02%; found C, 73.54%; H, 4.51%; N, 0.52%; \textbf{MASS}: m/z found for C_{17}H_{12}NO_3 was 278.3 ([M–1]).

2-(2-butyl-4-chloro-1H-imidazol-5-yl)-2,4-dihydro-1H-benzo[d][1,3]oxazine (3f):

This compound was obtained as black color solid in 86% yield. Melting point: 80-82 °C; \textbf{^1H NMR} (CDCl_{3}, 400 MHz): δ 8.2 (s, 1H, imidazole N-H), 7.60-6.60 (m, 4H, Ar-H), 5.61 (s, 1H, CH), 4.62 (s, 2H, -CH_{2}), 4.33 (s, 1H, -NH), 2.64 (t, 2H, CH_{2}), 1.60 (m, 2H, -CH_{2}), 1.33 (m, 2H, -CH_{2}), 0.92 (t, 3H, -CH_{3}); Elemental analysis calculated for C_{15}H_{18}ClN_{3}O: C, 61.75%; H, 6.22%; N, 14.40%; found C, 61.54%; H, 6.51%; N, 14.53%; \textbf{MASS}: m/z found for C_{15}H_{18}ClN_{3}O was 292.2 ([M+1]^+).

4-(7-chloro-2,4-dihydro-1H-benzo[d][1,3]oxazin-2-yl)phenol (3g):

This compound was obtained as brownish solid in 87% yield. Melting point: 139-141 °C; \textbf{^1H NMR} (CDCl_{3}, 400 MHz): δ 8.02 (s, 1H, Ar-H), 7.22-6.84 (m, 5H, Ar-H), 6.7-6.61 (d, 1H, Aromatic -CH), 5.24 (s, 1H, -CH), 5.06 (s, 1H, O-H), 4.81-4.72 (m, 2H, CH_{2}), 4.09 (s, 1H, NH); \textbf{^13C NMR} (CDCl_{3}, 100 MHz): 68.97, 94.73, 110.80, 115.66, 119.08, 129.97, 130.40, 133.39, 134.24, 139.12, 149.38, 159.12. Elemental analysis calculated for C_{14}H_{12}ClNO_2: C, 64.25%; H, 4.62%; N, 5.35%; found C, 64.14%; H, 4.21%; N, 5.53%; \textbf{MASS}: m/z found for C_{14}H_{12}ClNO_2 was 262.2 ([M+1]^+).

7-chloro-2-(1H-indol-3-yl)-2,4-dihydro-1H-benzo[d][1,3]oxazine(3h):

This compound was obtained as brownish solid in 85% yield. Melting point: 125-127 °C; \textbf{^1H NMR} (CDCl_{3}, 400 MHz): δ 11.80 (s, 1H, indole-NH), 8.64-8.01 (m, 3H, Ar-H), 7.50-7.07 (m,
5H, Ar-H), 5.10 (s, 1H, CH), 4.70 (s, 2H, -CH₂), 4.25 (s, 1H, N-H); 
Elemental analysis calculated for C₁₆H₁₃ClN₂O: C, 67.49 %; H, 4.60 %; N, 9.84 %; found C, 66.54 %; H, 4.51 %; N, 09.53 %; 
**MASS:** m/z found for C₁₆H₁₃ClN₂O was 285.8 ([M+H]⁺).

6-chloro-2-(2-phenyl-1H-indol-3-yl)-2,4-dihydro-1H-benzo[d][1,3]oxazine(3i):

This compound was obtained as colorless solid in 89% yield. Melting point: 220-222 °C; **¹H NMR** (CDCl₃, 400 MHz): δ 10.00 (s, 1H, N-H), 8.41-6.26 (m, 12H, Ar-H), 5.11 (s, 1H, -C-H), 4.72 (s, 2H, CH₂), 4.2 (s, 1H, -N-H); Elemental analysis calculated for C₃₅H₂₇N₂OCl: C, 73.23 %; H, 4.72 %; N, 7.76 %; found C, 73.34 %; H, 4.31 %; N, 7.43 %; **MASS:** m/z found for C₂₂H₁₇ClN₂O was 361.5 ([M+H]⁺).

3-(6-chloro-2,4-dihydro-1H-benzo[d][1,3]oxazin-2-yl)-4H-chromen-4-one (3j):

This compound was obtained as brownish solid in 83% yield. Melting point: 141-143 °C; **¹H NMR** (CDCl₃, 400 MHz): δ 8.55-7.77 (m, 7H, Ar-H), 4.71 (s, 2H, -CH₂), 4.65 (s, 1H, -CH), 4.12 (s, 1H, NH); Elemental analysis calculated for C₁₇H₁₂ClNO₃: C, 65.08 %; H, 3.86 %; N, 4.46 %; found C, 65.54 %; H, 3.51 %; N, 04.23 %; **MASS:** m/z found for C₁₇H₁₂ClNO₃ was 314.0 ([M+H]⁺).

6-methyl-2-(2-methyl-1H-indol-3-yl)-2,4-dihydro-1H-benzo[d][1,3]oxazine (3k):

This compound was obtained as light yellow color solid in 80% yield. Melting point: 102-104 °C; **¹H NMR** (CDCl₃, 400 MHz): δ 10.15 (s, 1H, N-H), 7.32-6.67 (m, 7H, Ar-H), 5.25 (s, 1H, -CH),
Chapter 2  
1,3-Benzoxazines and pyrido-1,4-oxazin-3-ones

4.6 (s, 2H, -CH₂), 2.5 (s, 3H, -CH₃), 2.40 (s, 3H, -CH₃); Elemental analysis calculated for C₁₈H₁₈N₂O; C, 77.67 %; H, 6.52 %; N, 10.06 %; found C, 77.14 %; H, 6.11 %; N, 10.43 %.

**MASS:** m/z found for C₁₈H₁₈N₂O was 279.2.

6-methyl-2-(2-phenyl-1H-indol-3-yl)-2,4-dihydro-1H-benzo[d][1,3]oxazine (3l):

![Chemical Structure](image)

This compound was obtained as colorless solid in 86% yield. Melting point: 220-222 °C; **¹H NMR** (CDCl₃, 400 MHz): δ 10.00 (s, 1H, N-H), 8.41-6.26 (m, 12H, Ar-H), 5.11 (s, 1H, -C-H), 4.78-4.67 (m, 2H, CH₂), 4.2 (s, 1H, N-H), 2.39 (s, 3H, CH₃). Elemental analysis calculated for C₂₃H₂₇N₂O; C, 81.15 %; H, 5.92 %; N, 8.23 %; found C, 81.34 %; H, 5.71 %; N, 8.43 %.

**MASS:** m/z found for C₃₃H₄₀N₂O was 341.2 ([M+1]+).

3-(6-methyl-2,4-dihydro-1H-benzo[d][1,3]oxazin-2-yl)-4H-chromen-4-one (3m):

![Chemical Structure](image)

This compound was obtained as yellow color solid in 88% yield. Melting point: 202-204 °C; **¹H NMR** (CDCl₃, 400 MHz): δ 8.5(s, 1H, O-CH), 8.3-6.6 (m, 7H, Ar-CH), 5.12 (s, 1H, C-H), 4.79-4.69 (m, 2H, -CH₂-), 4.13 (s, 1H, -C-H), 4.11 (s, 1H, N-H), 2.20 (s, 3H, -CH₃); Elemental analysis calculated for C₁₈H₁₅NO₅; C, 73.71 %; H, 5.15 %; N, 4.78 %; found C, 73.14 %; H, 5.11 %; N, 4.43 %; **MASS:** m/z found for C₁₈H₁₅NO₅ was 294.1 ([M+1]+).

2-(2-butyl-4-chloro-1-(4-nitrobenzyl)-1H-imidazol-5-yl)-2,4-dihydro-1H-benzo[d][1,3] oxazine (3n): Brown crystalline solid, Yield 81%, Melting point 53-55 °C; **¹H NMR** (DMSO-d₆, 400 MHz) δ 8.20-8.18 (d, J=8.4 Hz, 2H), 7.20-7.13 (m, 6H), 5.48 (s, 1H), 1.89 (s, 1H), 4.60 (s, 2H), 4.24 (s, 1H), 2.67-2.63 (t, J=8 Hz, 2H), 1.34-1.24 (m, 2H), 0.89-0.85 (t, J=7.2 Hz, 3H); **¹³C NMR** (100 MHz, DMSO-d₆): 147.81, 144.74, 143.21, 140.75, 135.21, 128.72, 128.01, 126.71,
123.10, 122.05, 120.95, 110.96, 92.30, 67.31, 46.69, 31.21, 26.31, 22.85, 14.68; Elemental analysis calculated for C_{22}H_{23}ClN_{4}O_{3}: C, 61.90 %; H, 5.43 %; N, 13.12 %; found C, 61.84 %; H, 5.41 %; N, 13.10%; Mass: m/z found C_{22}H_{23}ClN_{4}O_{3} 428.14 ([M+1]^+).

2.4.2. Biology

Rat intestinal acetone powder was purchased from Sigma Aldrich, St. Louis (USA), Acarbose was purchased from Glucobay Bayer AG (Germany). Glucose oxidase (GOD POD) kit was purchased from Piramal HealthCare Ltd, Mumbai (India). All other chemicals used were of analytical grade and purchased from Sigma Aldrich, USA and Sisco Research Laboratories, Mumbai (India).

2.4.2.1. Experimental animals

Adult Wistar rats weighing 150-180 g were collected from the University Central Animal Facility and housed a controlled environment. All animal experiments were approved by the Institutional Animal Ethical Committee (UOM/IAEC/08/2013 Dated: 28/09/2013), Department of Studies in Zoology, University of Mysore, Mysore and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.4.2.2. Effect of compound 3g and its combination with Acarbose on plasma glucose concentration by the oral maltose and sucrose tolerance test. The experimental animals were randomly divided into 11 groups each consisting of 5 rats. Following overnight fasting, animals were assigned to the following groups and treated with the respective compounds through oral gavage: Group - I Saline control (0.9% saline); Group - II Maltose control (3g/kg); Group - III Sucrose control (3g/kg); Group - IV Acarbose (3mg/kg) + Maltose; Group - V Acarbose
(3mg/kg) + Sucrose; Group - VI Compound 3g (50 mg/kg) + Maltose; Group - VII Compound 3g (100 mg/kg) + Maltose; Group - VIII Compound 3g (50 mg/kg) + Sucrose; Group - IX Compound 3g (100 mg/kg) + Sucrose; Group - X Acarbose (3mg/kg) + Compound 3g (50 mg/kg) + Maltose; Group –XI Acarbose (3mg/kg) + Compound 3g (50 mg/kg) + Sucrose. 5 minutes following compound 3g or Acarbose administration either maltose (3g/kg) or sucrose (3g/kg) solution were administered to the respective groups. Blood samples were collected from the tail vein to the tubes containing anticoagulant (2.5% trisodium citrate and 1.37% citric acid in the ratio 1:5; anti-coagulant: blood) at time point 0 (just before sucrose/maltose administration), and subsequently at 30, 60, 90, 120 and 180 min after substrate (sucrose/maltose) administration. Plasma was separated by centrifuging the samples at 2000 rpm for 10 min and stored at -20 °C until analysis. Plasma glucose concentration was determined by using the Glucose oxidase (GOD POD) kit according to the manufacturer’s protocol.

2.4.2.3. Determination of intestinal α-glucosidase inhibitory activity of Acarbose and its combination with compound 3g: Intestinal α-glucosidase activity was determined by measuring the amount of glucose hydrolyzed from maltose or sucrose [19]. Briefly, rat intestinal acetone powder was homogenized in 0.9% saline and the suspension was centrifuged at 10,000 g for 30 min at 4 °C and the supernatant obtained was used as enzyme source. The enzyme solution was pre-incubated with various concentrations of compound 3g (5-25 μM) or acarbose (5 μM) or combined [5 μM acarbose with increasing concentrations of compound 3g (1-10μM)] in 100 mM phosphate buffer pH 6.9 at 37 °C for 15 min and the reaction was started by adding maltose (37 mM) or sucrose (56 mM) and indubated at 37 °C for 30 min (for maltase) and 60 min (for sucrase). After the respective incubation period, reaction was terminated by keeping the samples in boiling water bath for 10 min. The concentration of glucose released from the
reaction mixtures was determined by using Glucose oxidase (GOD POD) kit according to the manufacturer’s protocol. Results were expressed as percentage inhibition of intestinal maltase/sucrase activity.

2.4.2.4. Determination of glucose uptake by diaphragm: Porcine diaphragm was purchased from a slaughter house, and cleaned using ice cold 0.9% saline several times to remove blood stains. This diaphragm was used for glucose uptake and inhibition by compound 3g. The diaphragm (100 mg) was suspended in a 24 well culture plate containing 500µl saline. In order to initiate the reaction, 5mM glucose was added to each well. To enhance the glucose uptake by the diaphragm, 1 unit of insulin was used in each well, and the volume was made up to 1 mL with saline. For inhibition studies, compound 3g at a concentration of 50 & 100 μM was used. Each well 100 µl of the assay mixture was aspirated at different time intervals (0, 5, 10, 20, 30 and 60 min). From this, glucose concentration was measured using Glucose oxidase (GOD POD) kit according to the manufacturer’s protocol.

2.4.2.5. Statistical analysis: Results are expressed as mean values ± SEM of three independent experiments. Data were compared by analysis of variance (ANOVA) followed by the Tukey “honestly significantly different” (HSD) post hoc analysis. Significance was accepted at \( p < 0.05 \) (*), \( p < 0.01 \) (**) and \( p < 0.001 \) (***)

2.4.2.6. Molecular modeling of compound 3g binding to the MGAM catalytic domain.

The software Insight II/Discovery Studio 2.5 from Accelrys was used for docking and visualization of the results as described earlier [20]. The crystal structure of amylase was retrieved (PDBID: 3TOP). Before performing the Ligand fit protocol of Discovery Studio, the protein was cleaned, and the size and spatial orientation of the active site was identified. All
energy calculations were performed using the CHARMM force field. Each energy-minimized final docking position of the ligands was evaluated using the interaction score function in the Ligand Fit module of Discovery Studio as reported previously [21].

2.4.2.7. Chem-informatic analysis

Utilizing the available amount of bioactivity data, we also rationalized the modes-of-action for the experimentally tested benzoaxazines using in silico target prediction approaches. To this end, the Parzen-Rosenblatt Window classifier was employed with the smoothing parameter set to 0.9, using approximately 190,000 bioactive compounds covering 477 human protein targets as the training dataset. For details on the method, dataset and validation see reference 18.

2.4.2.8. Inhibitory activity against α-Amylase

The α-amylase inhibition assay was performed according to a previous report [22]. Porcine pancreatic α-amylase (3 units/mL) was dissolved in 0.1 M phosphate buffered saline, pH 6.9. The various concentrations of 1,3-benzoaxazine derivatives (0–100 μM) were pre-incubated with enzyme independently for 10 min at 37 °C. The reaction was initiated by adding substrate solution (0.1% starch) to the incubation medium. After 10 min incubation, the reaction was stopped by adding 250 μL dinitrosalicylic (DNS) reagent (1% 3, 5-dinitrosalicylic acid, 0.2% phenol, 0.05% Na₂SO₃ and 1% NaOH in aqueous solution) to the reaction mixture. The reaction was terminated by keeping the reaction mixture in boiling water bath for 10 min. Thereafter, 250 μL of 40% potassium sodium tartrate solution was added to the mixtures to stabilize the colour. After cooling to room temperature in a cold water bath, the absorbance was recorded at 540 nm using a Varioskan multimode plate reader (Thermo Scientifics, USA). Acrabose was used as positive control. The percentage of inhibition was calculated using Abs Contol – Abs Sample
x100/Abs Control. Where Abs Control was the absorbance without sample, Abs sample was the absorbance of enzyme with compound. Concentration-response assays were used to determine the potency (IC$_{50}$) of 1,3-benzoxazine derivatives based on the logistic analysis of the concentration-response curve using Microsoft Excel.

2.4.2.9. Inhibitory activity against α-Glucosidase

The α-glucosidase inhibitory activities of synthesized compounds were evaluated using the method developed by Tsujii et al. [23]. Briefly, α-glucosidase was dissolved in phosphate buffer (50 mM, pH 6.9) and pre-treated with various concentrations of 1,3-benzoxazine derivatives (0–100 μM) independently for 10 min at 37 °C. The reaction was initiated by the addition of 50 μL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in phosphate buffer (50 mM, pH 6.9). The enzyme reaction was carried out at 37 °C for 30 min. The reaction was terminated by the addition of Na$_2$CO$_3$ (1 M). The enzymatic activity of α-glucosidase was quantified by measuring the absorption at 405 nm using a Varioskan multimode plate reader (Thermo Scientific, USA). The inhibitory effect of compounds was defined as inhibitory activity (%) = (Abs Control – Abs Compound treated)/Abs Control x100. Concentration-response assays were used to determine the potency (IC$_{50}$) of 1,3-benzoxazine derivatives based on the logistic analysis of the concentration-response curve using Microsoft Excel.
Chapter 2b: Pyrido-1,4-oxazin-3-ones

2.5. Results and discussion:

2.5.1. Chemistry: Since oxazine-based compounds have been reported to have good anti-oncogenic activity in hepatocarcinoma model and osteosarcoma model, we previously reported the anti-cancer activity of 1,3-oxazines [22] and here we prepared 2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one by reacting 2-aminopyridine-3-ol and chloroacetylchloride at 5 °C in basic medium and N-substituted pyrido1,4-oxazin-3-ones (Table 4) by reacting 2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one with various benzyl bromides in the presence of combustion derived bismuth oxide in DMF solvent (Scheme 31) The compounds obtained were characterized by melting point, \(^1\)H NMR, \(^{13}\)C NMR, and mass spectral analysis.

![Chemical Reaction Diagram]

Scheme-31: Schematic representation for the synthesis of N-substituted pyrido-1,4-oxazin-3-ones
Table 4: Library of synthesized N-substituted pyrido1,4-oxazin-3-ones.

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<th>Product 8 (a-j)</th>
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2.5.2. Cytotoxicity studies of Pyrido-1,4-oxazin-3-ones against lung cancer cells (A549):

The library of synthesized N-substituted pyrido-1,4-oxazin-3-ones were tested for its cytotoxicity against lung cancer cells (A549) using MTT assay, the results are summarized in table 5. Among the tested compounds, the compound 8e and 8h significantly inhibited the proliferation of lung cancer cells (A549), with an IC\textsubscript{50} values of 20.3 and 28.2 μM, respectively.

Table 5: Cytotoxicity data for the pyrido-1,4-oxazin-3-ones against A549 cancer cells.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>IC\textsubscript{50}(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td>NA\textsuperscript{a}</td>
</tr>
<tr>
<td>8b</td>
<td>NA</td>
</tr>
<tr>
<td>8c</td>
<td>20.3±0.12</td>
</tr>
<tr>
<td>8d</td>
<td>NA</td>
</tr>
<tr>
<td>8e</td>
<td>35.1±0.34</td>
</tr>
<tr>
<td>8f</td>
<td>37.2±0.14</td>
</tr>
<tr>
<td>8g</td>
<td>40.2±0.25</td>
</tr>
<tr>
<td>8h</td>
<td>28.2±0.10</td>
</tr>
<tr>
<td>8i</td>
<td>46.1±0.40</td>
</tr>
</tbody>
</table>
2.6. Conclusion:

We have identified a novel N-substituted pyrido[1,4-oxazin-3-ones as biologically active compounds against A549 lung cancer cells.

2.7. Experimental section

2.7.1. General procedure for the synthesis of N-substituted pyrido[1,4-oxazin-3-ones:

2.7.1.1. Preparation of combustion derived Bi$_2$O$_3$ (SCS-Bi$_2$O$_3$): We have prepared combustion derived Bi$_2$O$_3$ using reported protocol [23], Briefly, in a petridish, solution of Bi(NO$_3$)$_3$.5H$_2$O was prepared by dissolving 5 g Bi(NO$_3$)$_3$.5H$_2$O in 4 mL of 6 M HNO$_3$ and the excess acid was removed by heating on a hot plate, to this sucrose solution was added by dissolving 1.106 g of sugar in 25 mL of distilled water. An aqueous solution containing the above mixture of Bi(NO$_3$)$_3$.5H$_2$O was taken in a petridish. Excess water was allowed to evaporate by heating the solution on a hot plate until the formation of a yellow viscous gel. Then the petridish was introduced into a muffle furnace maintained at 400 °C. Initially, the viscous gel underwent dehydration and commenced smoldering combustion, which appeared at one end and propagated through the mass within one minute. A voluminous and porous nanocrystalline product was obtained.

The N-substituted pyrido[1,4-oxazin-3-ones were prepared in 2 steps,

2.7.1.2. Step 1: preparation of 2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (6):

The 2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one was prepared by using reported protocol [24], Briefly, A mixture of 2-amino-3-hydroxy pyridine, dichloromethane (10ml) and triethylamine is

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>8J</td>
<td>44.6±0.34</td>
</tr>
<tr>
<td>Paclitaxol</td>
<td>4.1±0.1</td>
</tr>
</tbody>
</table>

* - Not active
taken in round bottom flask, in ice cool condition, and then 1.5ml of chloroacetychloride was added drop wise and stirred. The crude product is obtained by evaporating dichloromethane.

2.7.1.3. Step 2: Preparation of N-substituted pyrido1,4-oxazin-3-ones (8):

In a 50 mL round bottom flask the 2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one(3), benzyl halide(4) and combustion derived bismuth oxide was added and stirred for 5 hrs in a DMF solvent at 40 °C, the reaction was monitored by TLC, after completion of the reaction the catalyst was filtered and water was added to the filtrate and extracted to ethyl acetate, washed with brine solution and dried with anhydrous sodium sulphate, then concentrated by using rotary evaporator and purified by column chromatography using ethyl acetate and hexane as eluent.

2.8. Spectral data of synthesized compounds:

4'-(3-oxo-2H-pyrido[3,2-b][1,4]oxazin-4(3H)-yl)methyl)-[1,1'-biphenyl]-2-carbonitrile (8a):

Yield 97%, melting point 134-136 °C. Elemental analysis calculated for C_{21}H_{15}N_{3}O_{2}: C, 73.89; H, 4.43; N, 12.31; found C, 73.64; H, 4.51; N, 12.23%; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400MHz) \delta: 8.04-8.02 (d, J=1.2 Hz, 1H), 7.74-7.71 (m, 1H), 7.60-7.56 (m, 3H), 7.48-7.40 (m, 4H), 7.24-7.22 (m, 1H), 6.95-6.92 (m, 1H), 5.40 (s, 2H), 4.72 (s, 2H). Elemental analysis calculated for C_{23}H_{32}ClN_{3}O_{2}: C, 66.09; H, 7.72; N, 10.05; found C, 66.14; H, 7.51; N, 10.13%; Mass: m/z found for C_{23}H_{32}ClN_{3}O_{2} was 342.2 (M+1)^{+}. 

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4-((6,6-dimethyl-4-phenyl-5,6-dihydro-4H-1,2-oxazin-3-yl)methyl)-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (8b):

Yield 94%, melting point 67-68 °C, Elemental analysis calculated for C_{20}H_{21}N_{3}O_{3}: C, 68.36; H, 6.02; N, 11.96; found C, 68.29; H, 6.09; N, 12.02%; \textbf{\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) } \delta: 7.95 (d, 1H), 7.5-7.1 (m, 6H), 4.6 (s, 2H), 4.55-4.45 (m, 1H), 3.5 (s, 2H), 2.05-1.80 (m, 2H), 1.25 (s, 6H). \textbf{Mass}: m/z found for C_{20}H_{21}N_{3}O_{3} was 352.22 (M+1)^+.

4-(4-isopropylbenzyl)-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (8c):

Yield 93%, melting point 78-79 °C. Elemental analysis calculated for C_{17}H_{18}N_{2}O_{2}: C, 72.32; H, 6.43; N, 9.92; found C, 72.25; H, 6.30; N, 10.02%; \textbf{\textsuperscript{1}H NMR(CDCl\textsubscript{3}, 400MHz) } \delta: 8.02-8.01(d, J=4.8 Hz, 1H, Ar-H), 7.39-7.37 (m, 2H, Ar-H), 7.20-7.12 (m, 3H), 6.92-6.89 (m, 1H), 5.31 (s, 2H), 4.68 (s, 2H), 2.86-2.82 (m, 1H), 1.20-1.18 (d, 6H). \textbf{Mass}: m/z found for C_{17}H_{18}N_{2}O_{2} was 283.18 (M+1)^+.

4-(4-(tert-butyl)benzyl)-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (8d):

Yield 94%, melting point 75-76 °C. Elemental analysis calculated for C_{18}H_{20}N_{2}O_{2}: C, 72.95; H, 6.80; N, 9.45; found C, 72.86; H, 6.71; N, 9.37%; \textbf{\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) } \delta: 8.03-8.01 (d, J=1.6 Hz, 1H), 7.40-7.38 (m, 2H), 7.30-7.28 (m, 2H), 7.21-7.18 (m, 1H), 6.92-6.89 (m, 1H), 5.32 (s, 2H), 4.68 (s, 2H), 1.26 (s, 9H); \textbf{Mass}: m/z found for C_{18}H_{20}N_{2}O_{2} was 297.20 (M+1)^+.

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4-(4-nitrobenzyl)-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (8c):

Yield 96%, melting point 68-69 °C, Elemental analysis calculated for C_{14}H_{11}N_{3}O_{4}: C, 58.95; H, 3.89; N, 14.73; found C, 58.82; H, 3.77; N, 14.61%. ¹H NMR (CDCl₃, 400 MHz) δ: 8.14-8.12 (m, 2H), 7.99-7.98 (d, J=1.6 Hz, 1H), 7.60-7.58 (m, 2H), 7.26-7.23 (m, 1H), 6.97-6.94 (m, 1H), 5.41 (s, 2H), 4.72 (s, 2H). Mass: m/z found for C_{14}H_{11}N_{3}O_{4} 286.12 (M+1)⁺.

4-((4-(4-chlorophenyl)-6,6-dimethyl-5,6-dihydro-4H-1,2-oxazin-3-yl)methyl)-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (8f):

Yield 95%, melting point 89-90 °C, Elemental analysis calculated for C_{20}H_{20}ClN_{3}O_{3}: C, 62.26; H, 5.22; N, 10.89; found C, 62.19; H, 5.15; N, 10.79%. ¹H NMR (CDCl₃, 400 MHz) δ: 7.90(d, 1H), 7.32-7.15 (m, 5H), 6.88-6.86 (m, 1H), 4.63 (s, 2H), 4.44-4.41 (m, 1H), 3.54 (s, 2H), 2.04-1.8 (m, 2H), 1.24 (s, 6H). Mass: m/z found for C_{20}H_{20}ClN_{3}O_{3} was 386.17 (M+1)⁺.

4-((3-oxo-2H-pyrido[3,2-b][1,4]oxazin-4(3H)-yl)methyl)benzonitrile (8g):

Yield 91%, melting point 59-60 °C, Elemental analysis calculated for C_{15}H_{11}N_{3}O_{2}: C, 67.92; H, 4.18; N, 15.81; found C, 67.85; H, 4.10; N, 15.75%. ¹H NMR (CDCl₃, 400 MHz) δ: 8.1 (d, 1H), 7.50-7.10 (m, 6H), 5.71 (s, 2H), 4.72 (s, 2H); Mass: m/z found for C_{15}H_{11}N_{3}O_{2} was 266.12 (M+1)⁺.
4-(cyclohexylmethyl)-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (8h):

Yield 93%, melting point 60-62 °C, Elemental analysis calculated for C_{14}H_{18}N_{2}O_{2}: C, 68.27; H, 7.37; N, 11.37; found C, 68.18; H, 7.29; N, 11.31%; \(^{1}H\) NMR (CDCl\(_3\), 400 MHz) \(\delta\): 8.00-7.99 (d, \(J=1.6\) \(Hz\), 1H), 7.21-7.19 (d, \(J=1.6\) \(Hz\), 1H), 6.92-6.89 (m, 1H), 4.64 (s, 2H), 4.02-4.00 (d, \(J=7.2\) \(Hz\), 2H), 1.85-1.82 (m, 1H), 1.69-1.57 (m, 5H), 1.18-1.14 (m, 3H), 1.07-1.01 (m, 2H). Mass: m/z found for C_{14}H_{18}N_{2}O_{2} was 247.1(M+1)^{+}.

2-(3-oxo-2H-pyrido[3,2-b][1,4]oxazin-4(3H)-yl)propylisoindoline-1,3-dione (8i):

Yield 95%, melting point 142-143 °C, Elemental analysis calculated for C_{18}H_{13}N_{5}O_{4}: C, 64.09; H, 4.48; N, 12.46; found C, 64.01; H, 4.40; N, 12.38%; \(^{1}H\) NMR (CDCl\(_3\), 400 MHz) \(\delta\): 8.1-8.0 (d, 1H), 7.9-7.7 (m, 4H), 7.4-7.3 (m, 2H), 4.70 (s, 2H), 4.6-4.5 (t, 2H), 4.4-4.3 (t, 2H), 1.8-1.7 (m, 2H); Mass: m/z found for C_{18}H_{13}N_{5}O_{4} was 338.1(M+1)^{+}.

4-(2,6-dichlorobenzyl)-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one (8j):

Yield 94%, melting point 100-102 °C, Elemental analysis calculated for C_{14}H_{10}Cl_{2}N_{2}O_{2}: C, 54.39; H, 3.26; N, 9.06; found C, 54.30; H, 3.31; N, 9.01%; \(^{1}H\) NMR (CDCl\(_3\), 400 MHz) \(\delta\): 8.2-8.1 (d, 1H), 7.6-7.2 (m, 5H), 5.2 (s, 2H), 4.6 (s, 2H), Mass: m/z found for C_{14}H_{10}Cl_{2}N_{2}O_{2} was 309.0(M+1)^{+}, 310.01(M+2)^{+}.
2.9. Biology

2.9.1. MTT Assay: The anti proliferative effect of the compounds synthesized against A549 (lung cancer) cells was determined by the MTT dye uptake method as described previously [25] Briefly, cancer cells (2.5x10^4/mL) were incubated in triplicate in a 96-well plate, in the presence of varying compound concentrations at a volume of 0.2 mL, for different time intervals at 37 °C. Thereafter, 20 µl MTT solution (5 mg/mL in PRS) was added to each well. After 2h incubation at 37 °C, a 0.1 mL lysis buffer (20% SDS, 50% dimethylformamide) was added; incubation was performed for 1 hour at 37 °C, and the optical density (OD) at 570 nm was measured by plate reader.
2.10. References:


Appendices
Chapter 2

1,3-Benzoxazines and pyrido-1,4-oxazin-3-ones

$^1$H NMR spectrum of compound 3a

Mass Spectrum of compound 3a
\(^{13}\)C NMR spectrum of compound 3a
$^1$H NMR spectrum of compound 3n

Mass spectrum of compound 3n
\(^{13}\text{C}\) NMR spectrum of compound 3n
$^1$H NMR spectrum of compound 8a
Mass spectrum of compound 8a
$^1$H NMR spectrum of compound 8d

Mass spectrum of compound 8d
\[ ^1H \text{ NMR spectrum of compound 8e} \]

\[ \text{Mass spectrum of compound 8e} \]