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FULL PAPER

Base Induced Condensation of Malononitrile with Erlenmeyer Azlactones: An Unexpected Synthesis of Multi-substituted Δ^2-Pyrrolines and their Cytotoxicity Activity

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Abstract: An efficient, metal free approach to synthesize multi-substituted Δ^2-pyrroline derivatives by mild base catalyzed cyclo-condensation of malononitrile with Erlenmeyer azlactones *via* 1,2 addition was developed. The modularity of this reaction was used to assemble a range of poly-substituted pyrrolines. Further, synthesized products were screened for cytotoxic properties on different cancer cell lines such as A549 (Human lung adenocarcinoma cells), HeLa (Human cervical adenocarcinoma cellls), Jurkat (Human chronic myeloid leukemia cells) and K562 (Human leukemic T cell Lymphoblast cells). Among the synthesized library of compounds **6f** and **6q** displayed potent cytotoxic activity.

Keywords: Erlenmeyer azlactones • Pyrrolines • Condensation reaction • 1,2 Addition reaction • Cytotoxicity

Introduction

Erlenmeyer azlactone is one of the most useful and important intermediates in organic synthesis.[1, 2] Erlenmeyer azlactones have been used in a wide variety of reactions as precursors for the generation of biologically active peptides, herbicides, fungicides, pesticides and agrochemical intermediates.[3] One of the most important reactions is conversion of glycine into different amino acids *via* azlactone intermediate.[4] Some other important reactions are aza-ene reaction with enamines,[5, 6] lactone ring oxygen replacement from other hetero atoms like *N* or *S*-containing substrates,[7] incorporation of allyl functionality in a selective manner to the Erlenmeyer azlactones core structure.[8] Alkylidene azlactones have been used in asymmetric hetero-Diels–Alder reactions, undergo reaction with isatins[9] and vinyl cyclopropanes,[10] which process through [4+2] annulations and [3+2]-cycloaddition respectively. Although, most reactions of Erlenmeyer azlactones rely on cycloaddition, few of them are concerned with their ring opening reactions. Among these, arylidene azlactone ring opening with amidines to access tetrasubstituted 1,3-diazinones through microwave-assisted transannulation reaction catalyzed by CsF has been reported.[11] In another study, indenones were synthesized by an intramolecular Friedel-Crafts reaction catalyzed by Keggin heteropolyacid $H_3PW_{12}O_{40}$ (supported on Al_2O_3) under microwave irradiation.[12] Further, ring opening of Erlenmeyer azlactones gave *anti*-α,β-diamino acid derivatives, an important building block for several bioactive molecules.[13] As Erlenmeyer azlactone is one of the precious intermediates, its synthesis and further exploration of its versality in organic synthesis is of interest to the scientific community. Therefore, we present an easy protocol to access this Erlenmeyer azlactones in acceptable yields. In addition, we explore a novel method for the synthesis of Δ^2-pyrrolines from Erlenmeyer azlactones.

Pyrrolines and pyrrolidine derivatives are ubiquitous structural motifs found in an array of natural products,[14, 15] amino acid derivatives[16, 17] and pharmaceuticals[18, 19] with diverse range of biological and medicinal properties. Pyrrolines are common structural frameworks present in porphyrin, which is essential components in haemoglobin, chlorophyll and cobalamin.[20, 21] This framework is also present in thienamycin, one of the most potent naturally produced antibiotics known so far.[22] Besides, pyrroline derivatives act as anticancer agents,[23] antibiotics,[24, 25] hepatitis C inhibitors[26] and antitubercular agents.[27] In organic synthesis, they have been used as key intermediates in total synthesis of natural products and to access other classes of important heterocycles.[28-30] Because of aforementioned natural, pharmaceutical and synthetic importance, development of an efficient synthetic method to access pyrrolines is intensively pursued by the scientific community.[31-33]

Most of the approaches describe the synthesis of Δ^3-pyrrolines, while less attention has been devoted towards synthesis of Δ^2-pyrrolines. The major successful routes, which yielded Δ^2-pyrrolines or 2,3-dihydropyrroles are Michael addition/cyclization

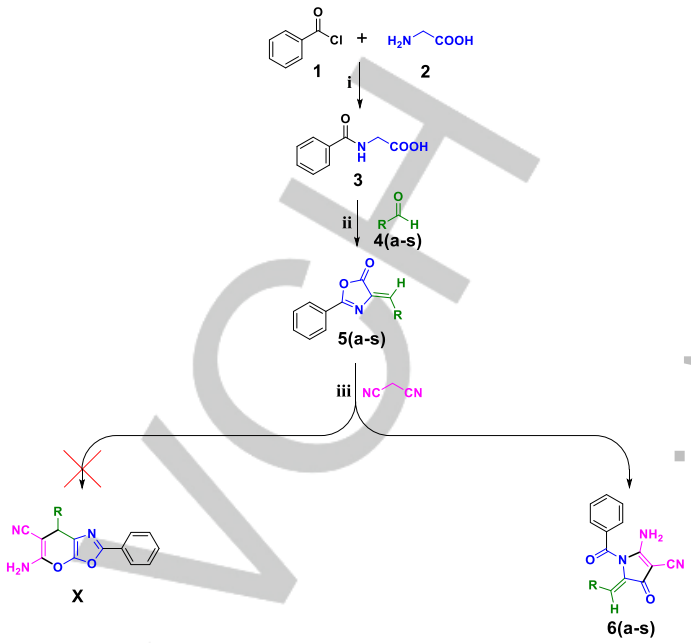
sequence[34, 35] and cyclization strategies in a one-pot sequential reaction.[36, 37] Other methods include the ring closing metathesis of enamides,[38] cyclization of sulfonamide anions with alkynyliodonium triflates,[39] ring expansion of aziridines,[40] hydroamination of alkynyl sulfonamides,[41] copper-catalyzed double *N*-alkenylation,[42] homopropargylamines reaction with I_2/AgOAc,[43] isomerization of 3,4-dihydropyrroles,[44] cycloadditions of nitro-olefins to isocyanoesters[45] and reactions of 1-cyano or 1-nitro cyclopropyl ketones with aniline.[46] In addition, a more convergent approach to synthesize Δ^2-pyrrolines is *via* [4+1][47-49] or [3+2][50, 51] cycloaddition reactions. The latter has been exploited with 1,3-oxazolium-5-oxides,[52] metallated azomethine ylides,[53, 54] isocyanoesters,[55, 56] and aziridines.[57, 58] Although, these methods are quite suitable for obtaining Δ^2-pyrrolines, there are several limitations. For example: use of costly organo (rosin-derived tertiary amine-thiourea),[35] metal (gold),[36] organometallics (iron[49] and silver complexes[50]) or phosphines[47] as catalyst, unstable substrates like sulfur ylides,[48] alkyl diazo acetate[49] and aziridines,[58] long reaction time[49, 51] and sophisticated reaction media like supercritical carbon dioxide,[57] etc. Thus, the development of a more efficient, cost-effective and metal free strategy with stable precursors for the rapid construction of structurally diverse Δ^2-pyrrolines is particularly appealing.

In continuation of our efforts to synthesize biologically important heterocyclic compounds,[59-64] we focused on fusion of oxazole five- and pyran six-membered heterocycles, which are common structural motifs in pharmaceutically active compounds[65, 66] and methods for their synthesis remain an active field in modern organic chemistry.[67] Very recently, Zamani *et. al.,* successfully developed a high-throughput methodology for the synthesis of fused chromeno[3,2-*d*]oxazoles starting from the same precursors *via* Erlenmeyer azlactones.[68] However, we failed to synthesize the fused pyrano-oxazoles due to unexpected Erlenmeyer azlactone ring opening reaction in the formation of Δ^2-pyrrolines.

We herein, present an easy way to access Erlenmeyer azlactones in acceptable yields and their unexpected ring opening reaction with malononitrile *via* cyclo-condensation to yield Δ^2-pyrroline derivatives catalyzed by base. Furthermore, it is demonstrated that two of the synthesized Δ^2-pyrroline derivatives, effectively reduced cell viability in A549, HeLa, Jurkat and K562 cancer cell lines.

Results and Discussion

Chemistry: The Δ^2-pyrroline derivatives were constructed from easily available starting materials, beginning from sequential azlactone formation and base induced 1,2-cyclo condensation reaction. *N*-benzoyl glycine/ hippuric acid (**3**) was prepared by reacting glycine (**2**) with benzoyl chloride (**1**). The prepared benzoyl glycine was reacted with ethyl chloroformate in the presence of triethylamine, followed by treatment with various aldehydes (**4**), resulted in the formation of functionalized Erlenmeyer azlactone precursors (**5a-s**) in 54-62% yields (Scheme 1). Selected Erlenmeyer azlactones (**5**) were cyclo-condensed with malononitrile in mild basic conditions resulted in the generation of multi-substituted dihydropyrroles (**6)**, instead of predicted pyrano[3,2-d]oxazoles **X** (Scheme 1).



Scheme 1. Unexpected synthesis of 2,3-dihydro pyrroline derivatives. Reagents and reaction conditions: i. (a) 5% NaOH, 2 hrs; (b) H_3O^+; ii. Cl-CO-OEt, NEt_3, toluene, 5 °C, 2 hrs; iii. NEt_3, ethanol, r.t., 30 min.

A reaction with benzaldehyde (**4a**), hippuric acid (**3**) and ethyl chloroformate was considered as model reaction to survey key parameters (temperature, solvent and base) to obtain Erlenmeyer azlactones (**5**). At 5 °C toluene and triethylamine were compatible and achieved higher yield compared to other standard organic solvents and bases (The compatibility of the solvents and bases are given in Supporting Information (Table S1)). Application of these conditions to a selected aryl/hetero aryl aldehydes and hippuric acid provided a variety of Erlenmeyer azlactones **5** in moderate to good yields (54-62%).

As a model reaction to achieve fused pyrano-oxazoles (**X**), we assessed the reaction of 4-benzylideno-5(4*H*)-oxazolone (**5a**) and malononitrile to survey key parameters of the optimal reaction process. In the solvents compatibility check, dimethyl formaldehyde (DMF), tetrahydrofuran (THF), acetonitrile and toluene with triethylamine base were not efficient and gave lower product yields of 28-63%. However, the solvents *viz.*, methanol and ethanol were gave higher product yield and found to be optimal at room temperature with respect to reaction time (30 minutes) and yields (72 and 81%). The same reaction at reflux temperatures of methanol and ethanol gave a product with 28 and 30% yields, respectively. Indicating that the reaction is more sensitive to changes in temperature and opting room temperature as the most favourable condition for this cyclo-condensation reaction. A survey of different bases showed the highest yield (81%) of product to be obtained with triethylamine. Thus, ethanol with triethylamine as a base at room temperature was found to be the optimal conditions to explore the substrate scope of the reaction.

The purified product formed was characterized by NMR and HRMS analysis, indicating pyrano[3,2-d]oxazole i.e. **X** (R = Ph) as anticipated. Surprisingly, single crystal X-ray diffraction (XRD)

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studies revealed the genuine structure of the product as **6a** ((*Z*)-2-amino-1-(benzenecarbonyl)-5-benzylidene-4-oxo-4,5-dihydro-1*H*-pyrrole-3-carbonitrile). The HRMS and NMR analysis could not distinguish between these two structures, since both of them are identical in their molecular weight and nuclei environments. Finally, with the help of single crystal X-ray diffraction we have elucidated the structure of unexpected product as **6a**. The ORTEP and packing diagram of **6a** is given in Figure 1. The crystallographic data is deposited at the **CCDC No. 1873630**.

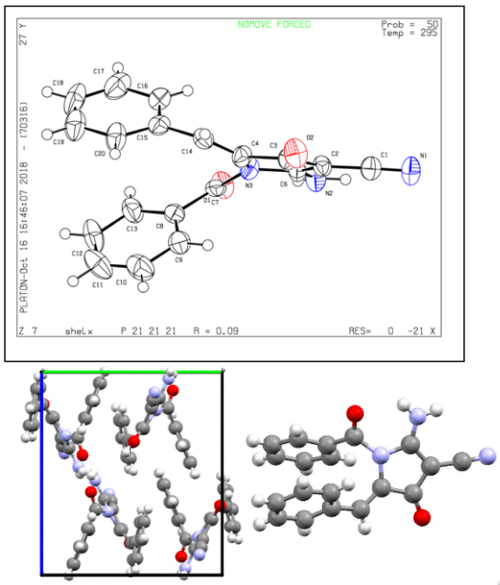
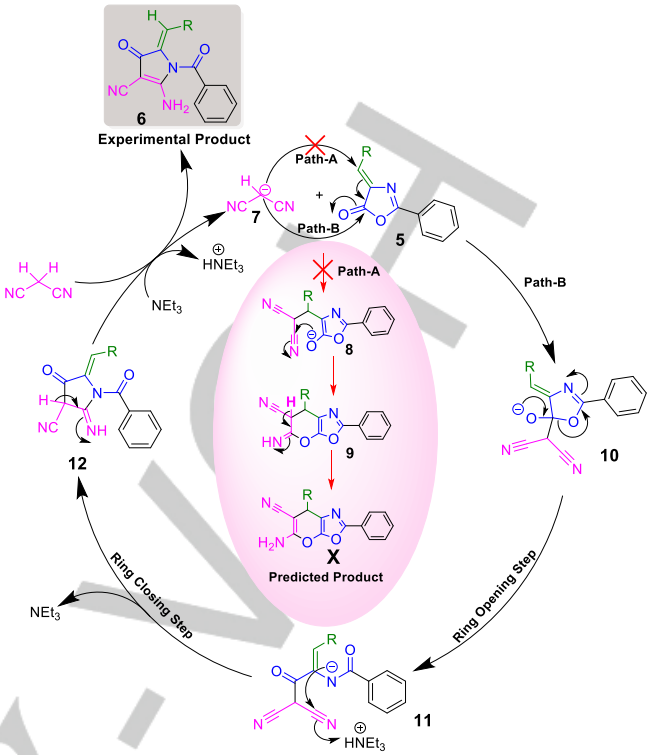


Figure 1. ORTEP and packing diagram of compound (*Z*)-2-amino-1-benzoyl-5-benzylidene-4-oxo-4,5-dihydro-1*H*-pyrrole-3-carbonitrile (**6a**), ($C_{19}H_{13}N_3O_2$)

The compound **6a** crystallized in orthorhombic space group $P2_12_12_1$. The lattice parameters are Cell: a = 10.548Å; b = 11.474Å; c = 12.946Å; α = 90°; β = 90°; γ = 90°. The final residual value, R1 is 0.09 with *π*-*π* stacking interaction running parallel to (010) direction. Further, XRD data revealed the geometrical isomerism of the exocyclic double bond as stable ***Z*** over ***E*** form.

Application of these conditions to a selected Erlenmeyer azlactones (**5**) and malononitrile provided a variety of Δ^2-pyrroline derivatives **6** (Table 1) in good to excellent yields (65−89%). Thus, various azlactones bearing halogens at different positions were able to yield corresponding products **6b-h** in 65-89% (Table 1). Similarly, Δ^2-pyrrolines tethered with electron-donating groups (methyl, methoxy, ethyl, isopropyl and phenoxy) formed respective products **6i-q** in 57-74% yield. Finally, azlactones derived from cinnamaldehyde and furfural afforded corresponding products **6r** and **6s** in 80 and 71% yield respectively. Almost all the substituents presented were well tolerated, different substituents on the aromatic aldehydes showing that the electronic effects of substituents had little influence on the reaction. However, Aldehydes with electron-withdrawing groups had a slightly faster reaction time and higher yield, compared to aldehydes containing electron donating groups.



Scheme 2. Plausible mechanism for the synthesis of Δ^2-pyrrolines.

Confirmation of genuine structure from XRD analysis, prompted us to identify the mechanism of the reaction to yield Δ^2-pyrrolines (**6**). Scheme 2 shows a suggested reaction mechanism consistent with the clean formation of Δ^2-pyrrolines. At first, The nucleophile (**7**) generated by the abstraction of acidic proton of malononitrile preferred to attack higher electropositive carbonyl carbon of azlactone (Path B; 1,2-addition) rather than addition to exocyclic double bond of the azlactone (Path A; 1,4-addition or Michael addition). According to our prediction *path A*, conjugate base of malononitrile **7**, attacks *β*-carbon of exocyclic double bond of **5** to form oxazol-5-olate **8**. This undergoes intramolecular cyclization to give pyrano[3,2-d]oxazole **X** *via* intermediate **9**. Whereas, experimental *Path B*, nucleophile **7** attacked carbonyl carbon of azlactone **5** to produce enamide **10**, which underwent intramolecular cyclization to generate Δ^2-pyrrolines **6** *via* electronic rearrangement through **11** and **12** (Scheme 2). The final product formed **6** has an exocyclic double bond, which is susceptible to undergo Michael addition with another molecule of malononitrile to give a new bicyclic compound. But in our reaction condition, the reaction stopped at the Δ^2-pyrroline step, even with use of excess of malononitrile and base. This is one of the added benefits of the herein developed methodology for the synthesis of Δ^2-pyrrolines tethered with an active exocyclic double bond.

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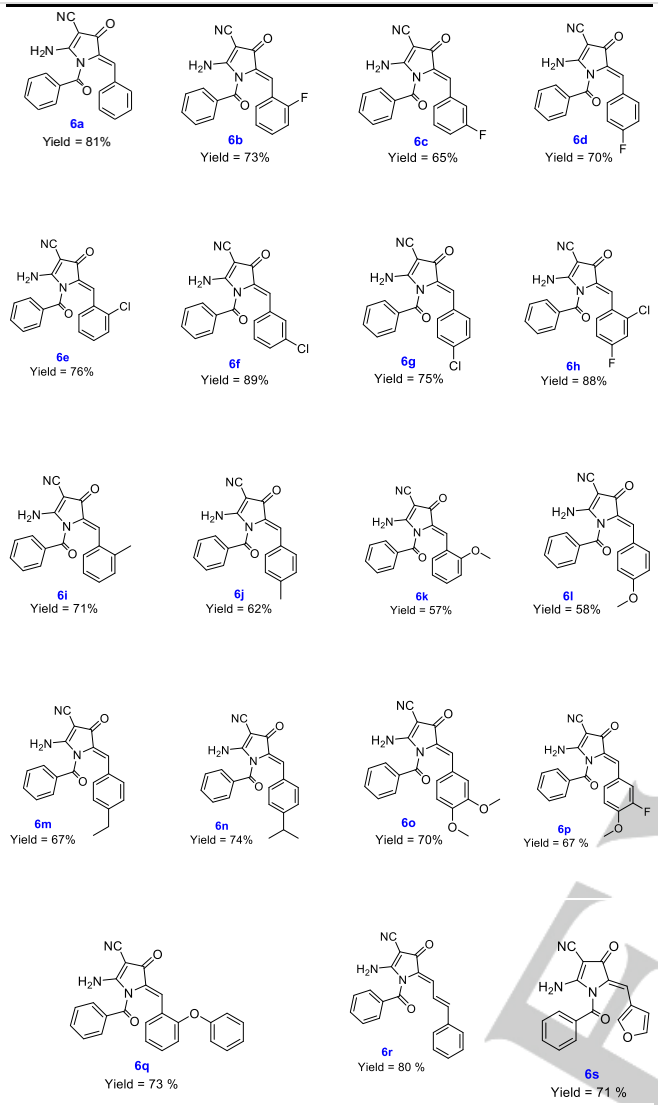
Table 1. Substrate Scope for Synthesis of Δ^2-Pyrroline Derivatives.



Biology: in vitro cytotoxicity assay

The synthesized novel Δ^2-pyrrolines (**6a-s**) were screened for their *in vitro* cytotoxic activity against A549 (Human lung adenocarcinoma cells), HeLa (Human cervical adenocarcinoma cells), Jurkat (Human chronic myeloid leukemia cells) and K562 (Human leukemic T cell Lymphoblast cells) through MTT assay. Results revealed that among the compounds treated **6e**, **6f**, **6h**, **6l**, **6o** and **6q** affected the viability of HeLa cancer cells. Where as, compounds **6f** and **6q** exhibited potent cytotoxic effect against all four cell lines (Fig 2). The compound **6q** is found to be more selective and has maximum cytotoxic effect on A549 cells with lower IC_{50} value of **6.23 μM**. Yet, it has good cytotoxic effect on HeLa and Jurkat cancer cell lines with respective IC_{50} values of **10.43** and **9.45 μM**. This selectivity could be attributed due to the presence of substituted *o*-phenoxy group on the phenyl ring of Δ^2-pyrroline.The compound **6f** substituted with *m*-chloro has almost equal activity on all the cancer cell lines selected *viz.,* A549, HeLa, Jurkat and K562 with respective IC_{50} values of **16.91**, **18.92**, **11.43** and **17.22 μM**. Position of chloro group on the phenyl ring of Δ^2-pyrroline (**6e**, **6f** and **6g**) has significant effect on cytotoxicity. Surprisingly, the chloro moiety at meta-position (**6f**) enhances the cytotoxic potential, while substitution on ortho-position (**6e**) results in decreased activity and substitution on para-position (**6g**) substantially diminishes the activity of the compound, reason for which is uncertain. The *in vitro* cytotoxic effect of active compounds **6f** and **6q** on cancer cell lines are depicted in Fig 2. Interestingly, both of the active molecules exhibited their cytotoxic effect in a dose and time dependent manner against the screened cancer cell lines.

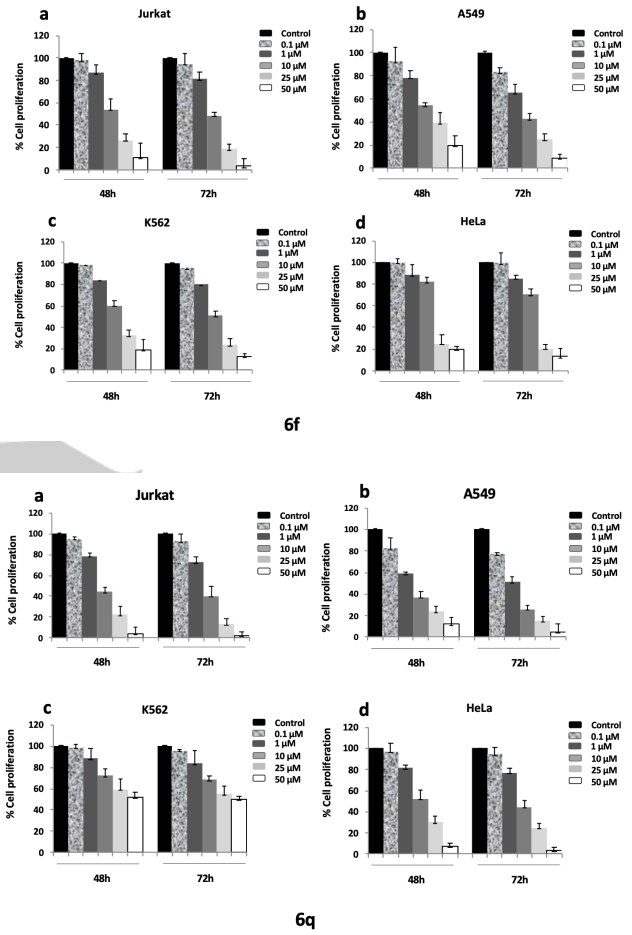


Figure 2. Cytotoxic effect of Δ^2-pyrrolines 6f and 6q on various cancer cells. Cells were treated with increased concentration of 6f and 6q (12.5 μM, 25 μM, and 50 μM) for 48h and 72h, cells were harvested and subjected for MTT assay to unveil the cytotoxic potential of compounds, DMSO treated cells served as vehicle control. Different cancer cells used for screening of 6f and 6q are Jurkat (a), A549 (b), K562 (c) and HeLa (d). Each experiment was repeated a minimum of three times and error bars indicate the SEM and P values compared with mean control groups with mean of 6f and 6q treated group, $p < 0.05$.

The rest of the synthesized Δ^2-pyrroline derivatives bearing different substitutions did not reduce viability in the cancer cell lines tested. Presence of active groups such as furan (**6s**) or a conjugated alkene (**6r**) on *C*-5 of Δ^2-pyrroline is not as effective as phenoxy substitution. It was observed from structural activity relationship studies, it appears that inductively electron-withdrawing chlorine at *m*-position (**6f**) or phenoxy substitution on *o*-position (**6q**) on the phenyl ring of Δ^2-pyrroline is essential for

cytotoxic activity. As of interest the hit compounds **6e**, **6f**, **6h**, **6l**, **6o** and **6q** were minimally cytotoxic to normal human embryonic kidney cells (HeK) at the tested concentrations (25 μM and 50 μM). The IC_{50} values of all the compounds are given in Supporting Information (Table S3).

Conclusion

In summary, we have developed a new, facile and practical metal-free synthetic route to novel multi-substituted and multi-functional Δ^2-pyrrolines in good to excellent yields. This protocol offers a promising, cost effective and simple approach to construct new medicinally and biologically important Δ^2-pyrrolines. Further, the synthesized library of compounds were screened for the cytotoxicity activity. Among the all, compounds **6f** & **6q** emerged as promising candidates without effecting human embryonic kidney (HeK) normal cells. Therefore, hit compounds **6f** and **6q** are worth studying further *in vivo* and is currently pursued in our laboratory.

Experimental Section

General Procedure for the Synthesis of Hippuric Acid:
Glycine (18.75g; 0.25 mol) was dissolved in 300 mL of 5% sodium hydroxide solution. To this, benzoyl chloride (32 mL; 0.275 mol) in five portions was added with stirring until benzoyl chloride completely reacted. The solution was transferred to the beaker and cooled by a few grams of crushed ice. Thereafter concentrated hydrochloric acid was added slowly with stirring until the mixture was acidic. The crystalline precipitate of benzoyl glycine was filtered and washed with cold water followed by diethyl ether. The solid product was collected, dried and recrystallized. Yield: 91%; M. P.: 85-86 °C.

General Procedure for the Synthesis of Erlenmeyer Azlactones:
Ethyl chloroformate (0.5844 mL, 6.1392 mmol) was added drop-wise to a mixture of hippuric acid (1 g, 5.5811 mmol) and Et_3N (0.78 mL, 5.5811 mmol) in dry toluene (10 ml) at 5-10 °C with constant stirring. After 30 minutes, Et_3N (1.6 mL, 11.1622 mmol) and aldehyde (6.1392 mmol) were added and cooled to 5 °C. The reaction mixture was allowed to stir for 4-5 hrs. After completion of the reaction monitored by TLC, the reaction mass was poured into ice cold water (100 mL). The mixture was extracted with ethyl acetate (3 x 20 mL), washed with H_2O (2 x 50 mL), brine (1 x 50 mL), dried over Na_2SO_4 and distilled under reduced pressure to give crude products, which were purified by column chromatography over silica gel using hexane-EtOAc (9: 1) as eluent or recrystallization with methanol whichever is permissible.

General Procedure for the Synthesis of Multi-substituted Dihydropyrroles:
Mixture of malononitrile (0.053 mL, 0.9628 mmol) and triethyl amine (0.12 mL, 8.8253 mmol) was added drop-wise to the selected azlactone (0.8023 mmol) in ethanol (6 ml) at room temperature with constant stirring. The reaction mixture was allowed to stir for 0.5 to 1 hr. After completion of the reaction monitored by TLC, the reaction mass was poured into ice cold water (20 mL). The mixture was extracted with ethyl acetate (3 x 10 mL), washed with H_2O (2 x 20 mL), brine (1 x 20 mL), dried over Na_2SO_4 and distilled under reduced pressure to give crude products, which were purified by column chromatography over silica gel using hexane-EtOAc (5 : 5) as eluent or recrystallization with chloroform whichever is permissible.

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Author Contribution Statement

Dr. S. M. Anil, Mr. N. Rajeev and Mr. K. R. Kiran were performed the experiments, analyzed the data and wrote the paper. Dr. M. S. Sudhanva and Dr. A. C. Vinayaka performed all biological experiments and analyzed results. Dr. S. M. Anil, Dr. T. R. Swaroop, Dr. R. Shobith and Dr. M. P. Sadashiva conceived and designed the experiments.

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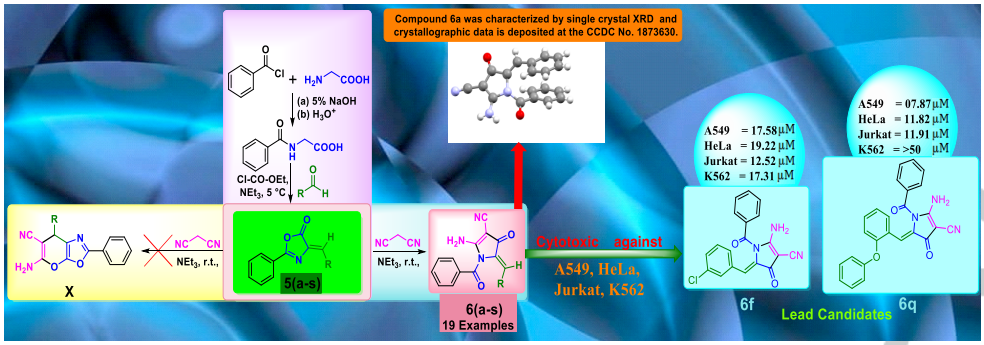

A mild base catalyzed cyclo-condensation of malononitrile with Erlenmeyer azlactones *via* 1,2 addition to yield Δ^2-pyrroline derivatives was developed. Further, synthetic library of compounds were screened for cytotoxic activity against A549, HeLa, Jurkat and K562 cancer cell lines.