Design, synthesis and evaluation of small molecules as TLR9 antagonist

1. Introduction:

The term TLR is derived as this receptor was first identified in Drosophila Toll, which show anti-microbial defences. TLRs are the trans membrane proteins that recognize distinct pathogen-associated molecular patterns [1].Thirteen mammalian TLR are identified, among them expression of 10 is known in humans. These are expressed on cells of innate immune system including monocytes, macrophages, myeloid cells such as neutrophils, dendritic cells (DCs), and NK cells. Some TLRs are found on endothelial cells, B lymphocytes, T -cell subsets, including regulatory T cells and some are intracellular.

The TLR family mainly divided into two subgroups, Depending on their cellular localization, extracellular and intracellular. TLR1, 2, 4, 5, 6, and 10 are largely localized on the cell surface to recognize pathogen associated molecular pattern (PAMPs). Conversely, TLR3, 7, 8, and 9 are localized in intracellular organelles such as endosomes / lysosomal compartments and the endoplasmic reticulum. TLR-2 forms heterodimers with TLR-1 and TLR-6 that recognize bacterial proteins and lipoteichoic acid. TLR-5 recognizes bacterial flagellin and TLR-4 recognizes endotoxin or lipopolysaccharide (LPS) on gram-negative bacteria.TLR-3 binds to double stranded viral RNA and there is some evidence that TLR-8 can activated by single-stranded RNA. TLR-7 recognizes single stranded RNA and TLR-10 ligands has not identified.

TLR ligands vary in binding bacterial cell wall components, bacterial genome fungal, parasitic, and viral products and synthetic analogues of natural products that have unusual molecular motifs. TLRs can also bind auto (self) molecules such as heat shock proteins (HSPs) and mammalian genomic DNA. Usually the host-derived ligands are shielded from the immune system and their emergence, for example after tissue trauma, signals that intervention by the immune system is required. TLRs help the immune system to fight the dangerous, protect the useful and neglect the vast majority of harmless microorganisms that colonizes our bodies. From integrity hypothesis proposes three functions for TLRs. The first is to detect unusual molecular patterns. The second is to sense the extent of tissue damage and the third is to determine the class of immune response. Specialized cells of central immunity such as dendritic cells and T and B cells are principle players in integrating these TLR signals into a specific immune responses.

Receptor	Ligand	Ligand location	Adapter (s)	Location	Cell types
TLR1	lipopeptides	Bacterial lipoprotein	MyD88/MAL	Cell surface	Monocytes B lymphocytes Dendritic cells.
TLR 2	Lipoproteins, lipoteichoic acid	Bacterial peptidoglycans,	MyD88/MAL	Cell surface	Monocytes Mast cells Myeloid dendritic cells
TLR3	Double stranded RNA	viruses	TRIF	Cell compartment	Dendritic cells B lymphocytes
TLR 4	Heparin sulfate fragments	Host cells	MyD88/MAL/TRIF/TRAM	Cell surface	Monocytes Myeloid dendritic cells
TLR 5	Profilin	Toxoplasma gondii	MyD88	Cell surface	Monocytes/macrophages Dendritic cells
TLR6	Diacyl lipopeptides	Mycoplasma	MyD88/MAL	Cell surface	Monocytes Mast cells B lymphocytes
TLR 7	Imidazoquinoline	Small synthetic compounds	MyD88	Cell compartment	B lymphocytes Plasmacytoid dendritic cells
TLR 8	Small synthetic compounds;		MyD88	Cell compartment	Monocytes mast cells, dendritic cells
TLR 9	Unmethylated CpG oligodeoxynucleotide DNA	Bacteria, DNA viruses	MyD88	Cell compartment	Plasmacytoid dendritic cells Monocytes

Table 1: Information about different TLR

1.2 TLR signalling:

TLRs signalling process follow two separate pathways. One is MyD88- dependent pathway and another one is MyD88 independent pathway. MyD88-pathway that leads to the production of inflammatory cytokinines. MyD88-independent pathway associated with the stimulation of INF-B and maturation of dendritic cells by the activation of PAMPs or DAMPs, the TLRs inducing the recruitment of adaptor proteins through cytoplasmic TIR domain. Adaptor proteins contain a variety of protein-binding modules that link protein-binding partners together and facilitate the creation of larger signalling complexes. Specificity in signalling would be achieved by the type of protein binding modules encoded by the adaptor protein. These proteins are positioned to regulate cell signalling in a spatial and temporal fashion (Daniel C Flynn 2001).

The adaptor proteins include the TIR domain containing proteins. That 5 adaptor proteins are linked to the TIR domain. They are MyD88, Mal (MyD88 adaptor like), TIRAP (TIR domain containing –adaptor proteins), TRIF (TIR domain containing inducing interferon-beta), TRAM (TRIF related adaptor molecule).

The recruitment process of adaptor proteins by the MyD88 involves in instantly it recruits the IRAK1 and IRAK4.IRAK4 activates the IRAK 1 by the phosphorylation .After activation of IRAK1,both IRAK1 and IRAK 4 both leave the MyD88-TLR complex. They both are temporally associated with the TRAF6, which leads to TRAF6 ubiquitination. Then there are two oligomers they are BCI 10 and MALT1forms they bind to the TRAF6 and promoting TRAF6 self- ubiquitination.IRAK2 play a vital role in ubiquitination TRAF6.for ubiquitination TRAF6 forms a complex with the TAB2/TAB3/TAK1 inducing TAK 1 activation .then TAK1 binds to the IKK complex which includes the scaffold protein NEMO, which leads to the phosphorylation of IkB and subsequent nuclear localization of NF-kβ activation. Activation of NF-kβ triggers the production of pro inflammatory cytokines such as TNF and IL-1 and IL-12.

Each TLRs induces different signalling responses by using different adaptor molecules .For the signalling of TLR-2and TLR-4 requires two adaptor molecules they are TIRAP/MAL, which is involves the MyD88-dependent pathway. For the TLR-3 signalling requires two adaptor molecules they are TRIF/TICAM-1and TRAM/TICAM-2, which triggers the INF-β in a response to double-stranded RNA, in a MyD88-independent pathway this function is restricted to the TLR-4.

TLR3, TLR7.TLR8, and TLR9 recognize by the viral nucleic acids, and induce type-1 INFS. the induced the type-1 INFs may differ depends on the activation of TLR. They involves in the regulation of interferon factors, IRFs, a family of transcription factors play a vital role in viral defense, cell growth and immune regulation. Three IRFs(IRF3,IRF5 ,IRF7) which are act as a direct transducers of virus- mediated TLR signalling.TLR3 and TLR-4 activates the IRF-3 and IRF-7,while TLR7 and TLR-8 activates IRF-5 and IRF-7.type-1 IFN production stimulated by the TLR-9 ligand, CPG-A has been shown mediated by the PI(3)k and m TOR. Robust TLR7/9 responses to self-nucleotides predispose to autoimmune diseases. Nucleotides are sensed by TLR7/9 in endolysosomes whereas self-derived nucleotides are degraded before reaching endolysosomes and stimulate TLR7/9. This discrimination may be broken in inflammatory or autoimmune situations, where a variety of nucleotide-binding proteins such as autoantibodies, anti-microbial peptides and high mobility group are complexed with host nucleotides and proteins may become resistant to degradation reach the endolysosome and stimulate TLR7/9 leading to an autoimmune reaction.

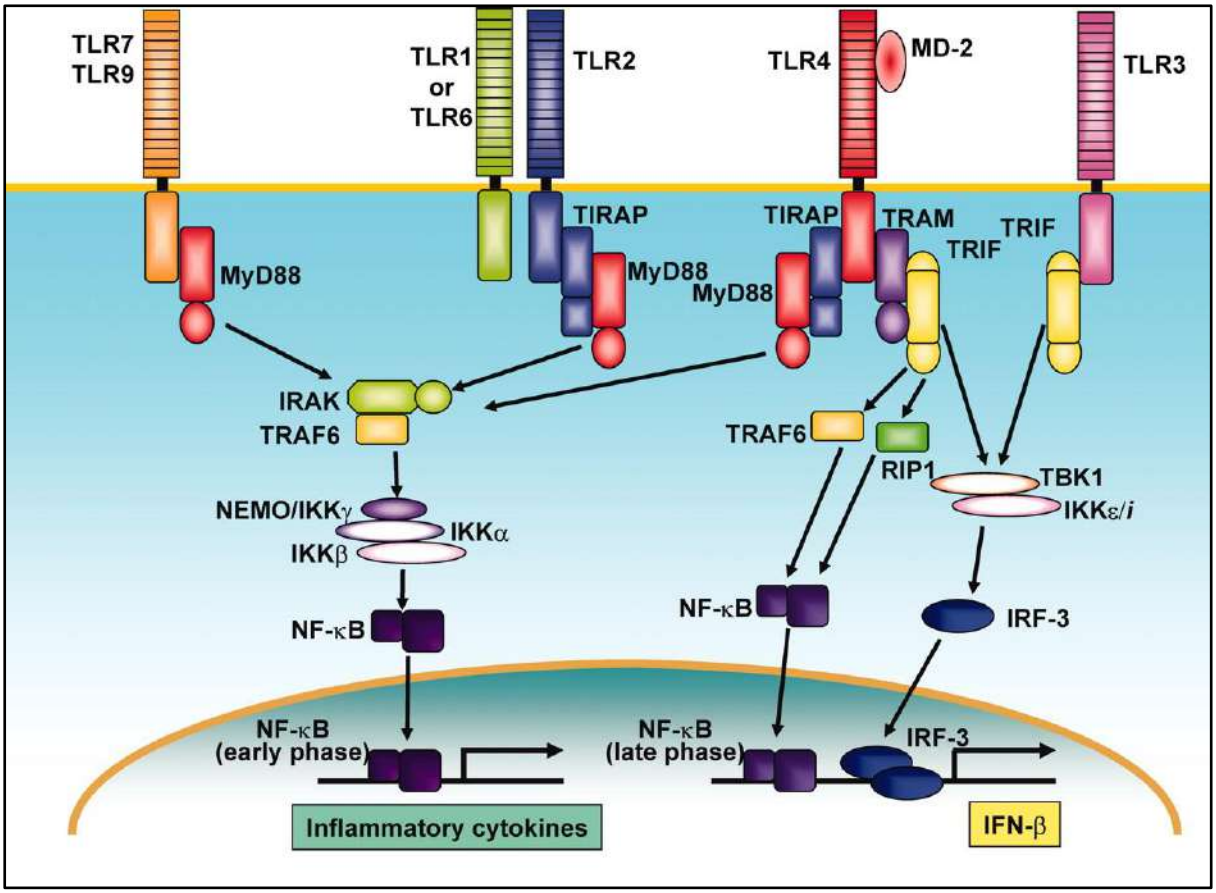


Figure 1: Signalling pathway of different TLR

1.3 Localization of TLR9 regulates its responses:

The subcellular distribution of TLR9 and its ligand influences not only the strength of responses but also types of responses. TLR9 senses unmethylated CpG DNA, which is abundant in bacterial and viral DNA. The CpG DNA is internalized into early endosomes and is subsequently transported too lysosomes via a clathrin-dependent pathway and then TLR9 recognizes the internalized CpG DNA in endosomes and lysosomes. The translocation of TLR9 into endosomes and lysosomes is one of the essential regulatory steps for TLR9 activation, because the compartment for ligand binding and signal transduction is not ER but rather CpG DNA containing- endolysosomes. In endolysosomes, acidification and protease activity are essential for the binding of unmethylated CpG with TLR9 and subsequent TLR9 activation. In endosomes and lysosomes, TLR9 can transmit signals in a MyD88 adapter-dependent fashion. CpG DNA stimulation can strongly induce type I IFNs. Interestingly, CpG DNAs are classified into two main groups, A-type CpG DNA (CpG-A) and B-type CpG DNA (CpG-B). The former, which mainly elicits type I IFM a／b production, contains a single CpG motif and a poly-G tail at the 3-end on a mixed phosphorothioate–phosphodiester backbone. The latter, which activates DCs and macrophages to produce proinflammatory cytokines, contains single or multiple CpG motifs on a phosphorothioate backbone. CpG-B also induces B-cell proliferation and antibody production. In pDCs, there are two different signaling mechanisms to respond to the different types of CpG DNA. The CpG-B rapidly traffics through early endosomes into late endosomes or lysosomes, whereas CpG-A are retained for longer period of time in early endosomes of pDCs. The prolonged retention of CpG-A provides a platform for extended activation of the signal transduction complex, consisting of MyD88 and IRF7, which leads to robust type I IFN production. DCs seem to detect different types of DNA in early endosomes or in late endosomes and lysosomes, the former is coupled with IRF7 activation and type I IFN and the later it is coupled with proinflammatory cytokine production.

1.4 TLR9- as a therapeutic target:

TLR 9 is a member of nucleic acid sensing TLR subfamily and expressed in the ER. The C-terminal fragment of TLR9 is competent to bind DNA from microbes that traffic to endosomal compartments and activate downstream pathways. But recent evidence suggests that the N-terminal also plays an important role in ligand binding and signalling. Recently published data shows that the TLR9-CTD exhibits the unique feature of extended aromatic

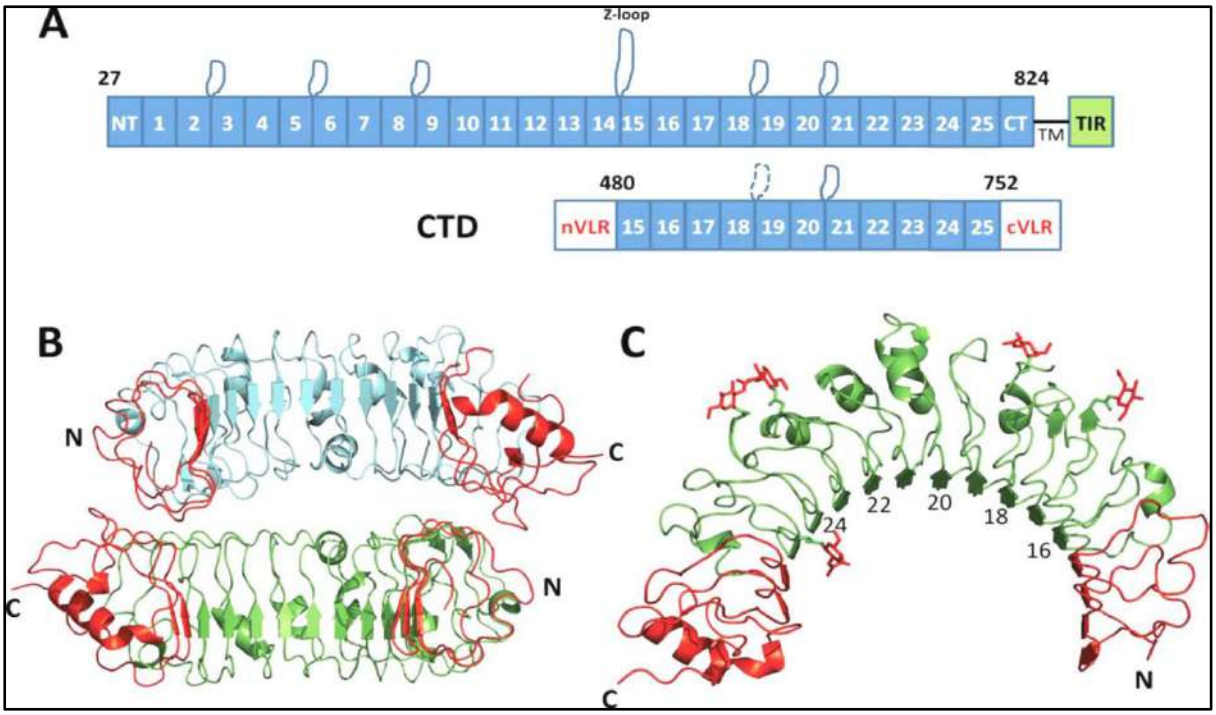


Figure 2: Crystal structure of TLR9 ECT domain (A)-sequence of ECT domain of TLR9 (B) Crystal structure of dimer TLR (C) crystal structure of ECT domain

side chain stacking on its concave face. Thus, as TLR9 is structurally similar to TLR8, it presumably binds DNA in a manner similar to dsRNA binding by TLR3. Studies showing that self-recognition by the innate immune system can lead to autoimmune diseases. Evidences support that overproduction of IFN-α in autoimmune disease like SLE is due to self-recognition of nucleic acid sensing TLRs. Inflammation in the absence of pathogens can be induced by molecules released by apoptotic cells, such as high mobility group box 1 (HMGB1), or by chemicals that provoke tissue injury. Endogenous ligands released from damaged tissues or apoptotic cells have been identified for all human TLRs except TLR5 and TLR10.18, 19 TLR2 and TLR4 trigger signalling by the recognition of heat shock proteins and TLR4 also responds to extracellular matrix fragments, fibrinogen, and b-defensin. TLR3 gets activated through mRNA recognition. Studies shows that TLR7 and TLR9(expressed by human PDCs) stimulation by DNA-anti-DNA immunoglobulin G (IgG) immune complexes (ICs) leads to very high levels of IFN-α production by PDCs. Exogenous viruses acting through these TLRs would be expected to induce IFN-α and thus exacerbate the SLE. IFN-α is a strong promoter of B-cell differentiation into antibody-producing plasmocytes and can induce B-cell survival factors such as BAFF [B-cell activating factor belonging to the tumour necrosis factor (TNF) family]. This leads potentially to a positive feedback loop in which

antibodies produced by auto reactive B cells can activate IFN-α from PDCs, which in turn is promotes B-cell survival, activation, and differentiation. TLR-induced IFN-α has also been linked to other diseases such as psoriasis and atherosclerosis.

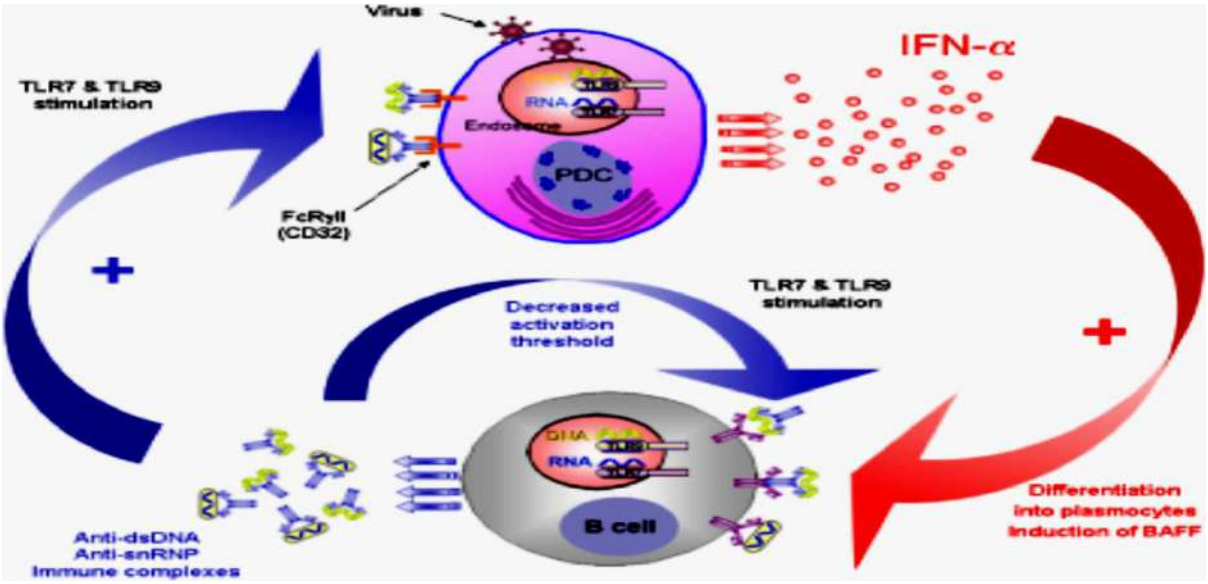


Figure 3: TLR9 and autoimmune

TLRs act as double action switch. Either promoting or inhibiting the disease progression. The therapeutic agent which target the TLR must be able to antagonise the harmful effects without affecting host defense function potential of harnessing directing the innate immune system and drugs targeted to the TLR, to prevent the human inflammatory and auto immune diseases as well as cancer, appears to be promising.

2. Aim and objectives:

Toll like receptors are the most widely expressed receptors in innate immune system and are the first defenders of pathogen attack. TLR9 recognize bacterial and viral DNA. Studies have been shown that unregulated activation of immune system through TLR plays a crucial role in autoimmune diseases and inflammatory disorders. Hence there is a wide scope to develop target specific therapies, without affecting normal pathogen defense. Current project aimed at identifying new small molecules as TLR9 antagonists, determine their potential inhibition of TLR9 activation and explore their SAR. we have derive purine derivative from known antagonist resiquimod by logical minimization of structure as describe in figure.

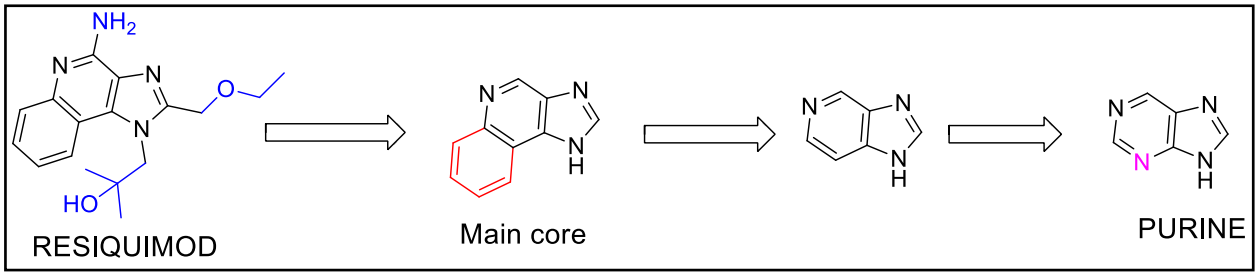


Figure 4: Generation of purine from Resiquimod

Close observation of literature reported antagonist of TLR9 revealed that inclusion of strong base in side chain increase the activity of antagonist. Previously we have checked some disubstituted purine analogue those are not shows proper activity so we focus on Trisubstituted purine analogue (figure)

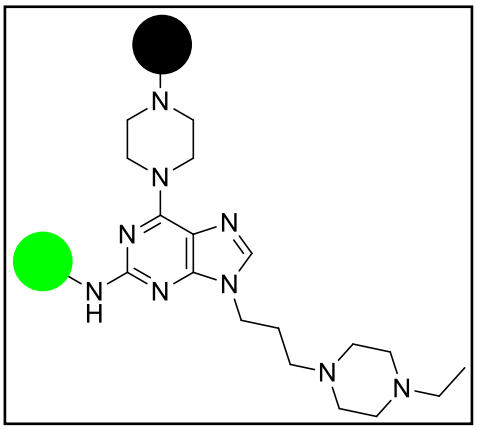
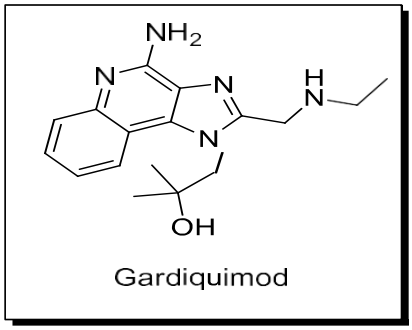


Figure 5: our modified substitution site of purine scaffold

3. Review of literature:

3.1 TLR9 antagonists:

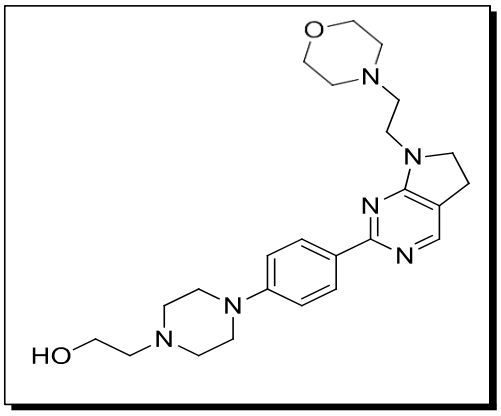


Gardiquimod

3.1.1 Imidazoquinolines as TLR9 antagonists: Imidazoquinolines, which are TLR7/8 agonists, inhibit TLR9 and TLR3 even in the absence of TLR7 or TLR8, and their mechanism of inhibition is similar to the antimalarial. Studies shows that Gardiquimod and Resiquimod inhibit TLR9 signallingdirectly.

3.1.2 Dihydropyrrolo[2,3-d]pyrimidines:

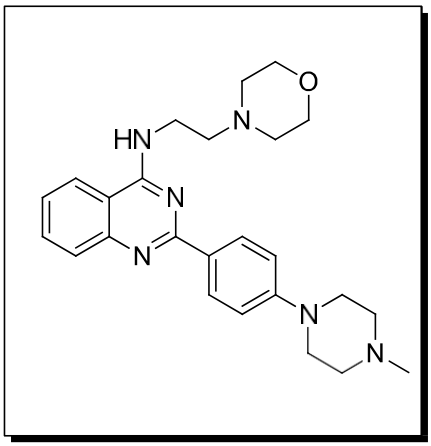
Manabu Watanabe et al. identified Dihydropyrrolo[2,3-d]pyrimidines as novel TLR9 antagonists and also reported that a structure-activity relationship study of a known TLR9 antagonist led to the promising compound 18, which showed potent TLR9 antagonistic activity, sufficient aqueous solubility for parenteral formulation, and druggable properties.

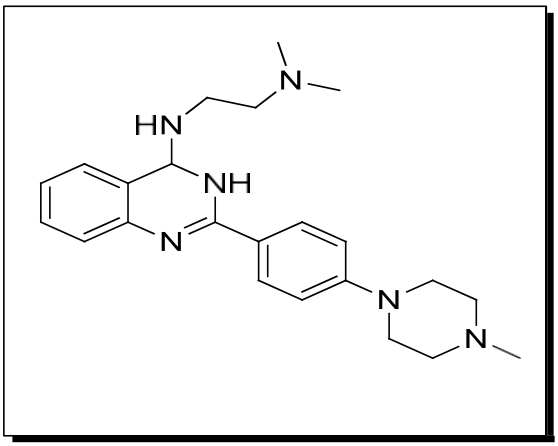


3.1.3 Quinazolines:

Disubstituted quinazolines as TLR9 antagonists were first reported by Lipford and claimed to be useful in treatment of autoimmune diseases, transplant rejection, and sepsis. Lipford

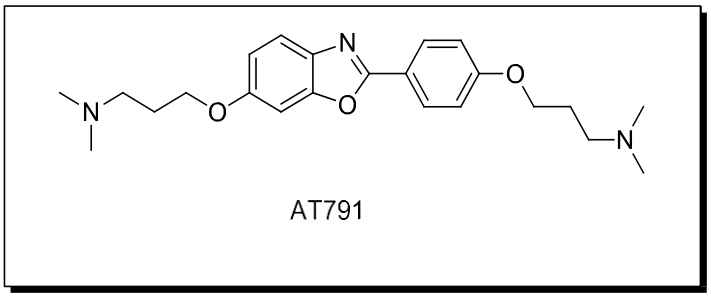
reported extensive SAR studies along with biological activity. The following few are best combined elements of SAR showing biological activity at single digit nano molar range.[35,36]





3.1.4 Benzoxazoles:

Benzoxazoles were introduced as novel TLR9 antagonists by Lamphier et al and identified two molecules inhibiting DNA-TLR9 interaction invitro.[38]



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3.2 TLR9 Agonists:

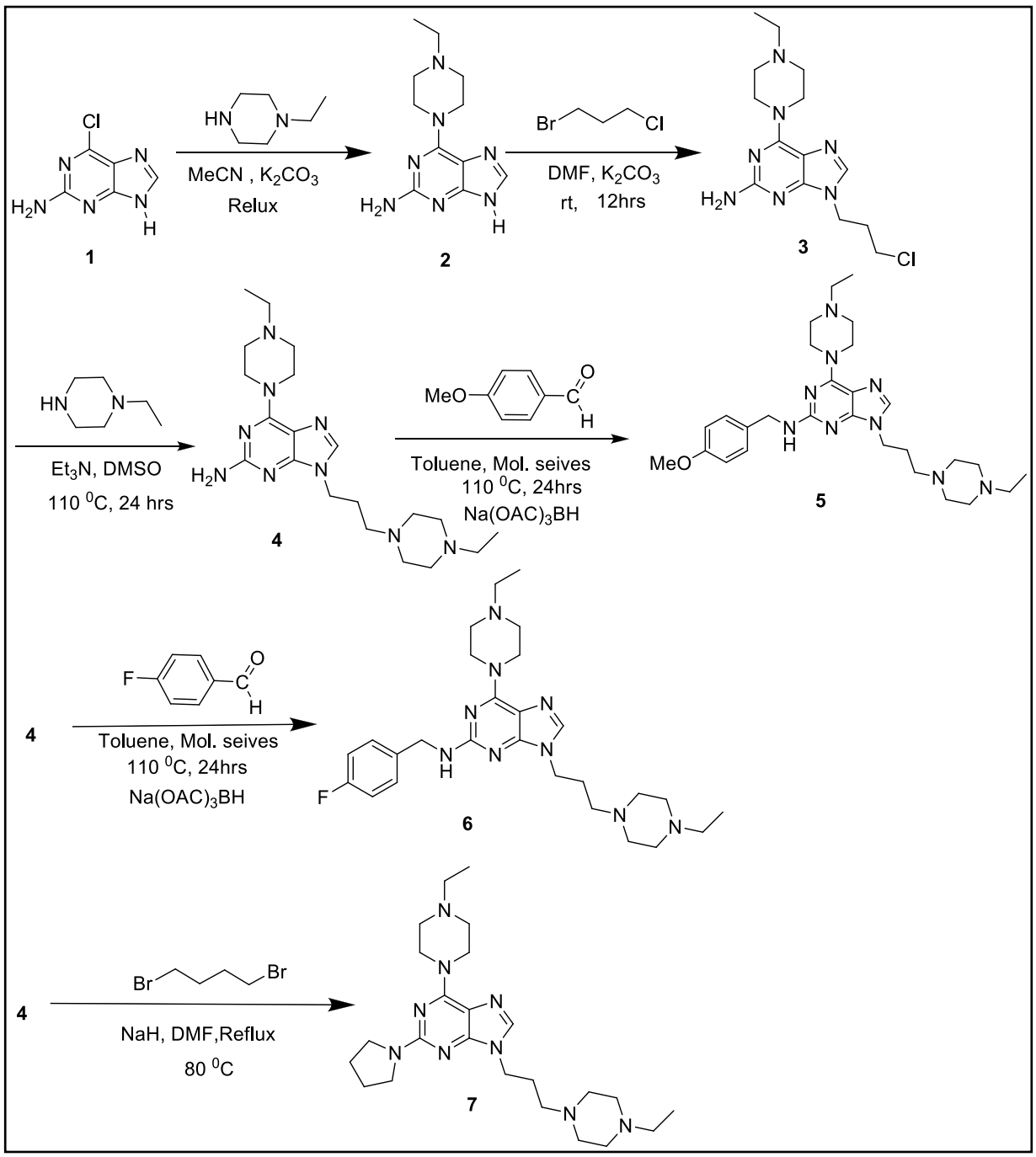
Agonists like synthetic Dimeric phosphorothioate 11-mer oligonucleotides, linked with a glycerol bridge, which incorporate unnatural synthetic CpG motifs were reported by Yu et al. from Idera Pharmaceuticals. They have studied SAR of those agonists.[30] M. Kreig from coley pharmaceutical group also identified some synthetic Oligodeoxynucleotides as TLR9agonists.[31]

1. ODN 2216-GGgggacgatcgtcgGGGGG
2. CPG 7909-TCGTCGTTTTGTCGTTTTGTCGTT
3. ODN 2395-TCGTCGTTTTCGGCGCGCGCCG

4. Materials and methods:

All starting materials and reagents purchased commercially from suppliers. Normal solvents and dry solvents also purchased from the suppliers.. TLC was performed on silica gel plates, and the spots were visualised under UV light (254 nm and 365nm). ^{1}H NMR were recorded at 300 MHz (Brucker-DPX) and 600 MHz (Brucker-Avance) frequency. ^{1}H, ^{13}C spectra were recorded using $CDCl_3$, CD_3OD and Acetone-d_6 as solvents as per the requirement and also using TMS as internal standard. Chemical shifts were measured in parts per million (ppm) referenced to 0.0 ppm for Tetramethylsilane. The following abbreviations were used to explain position: s = singlet, d = doublet, t = triplet, m = multiplet, br. = broad. Coupling constants, *J* values were reported in Hertz unit (Hz).HRMS (m/z) were measured using EI and ESI techniques (JEOL-JMS 700 and Q-Tof Micro mass spectrometer respectively). Melting points were taken in Digital melting point apparatus.

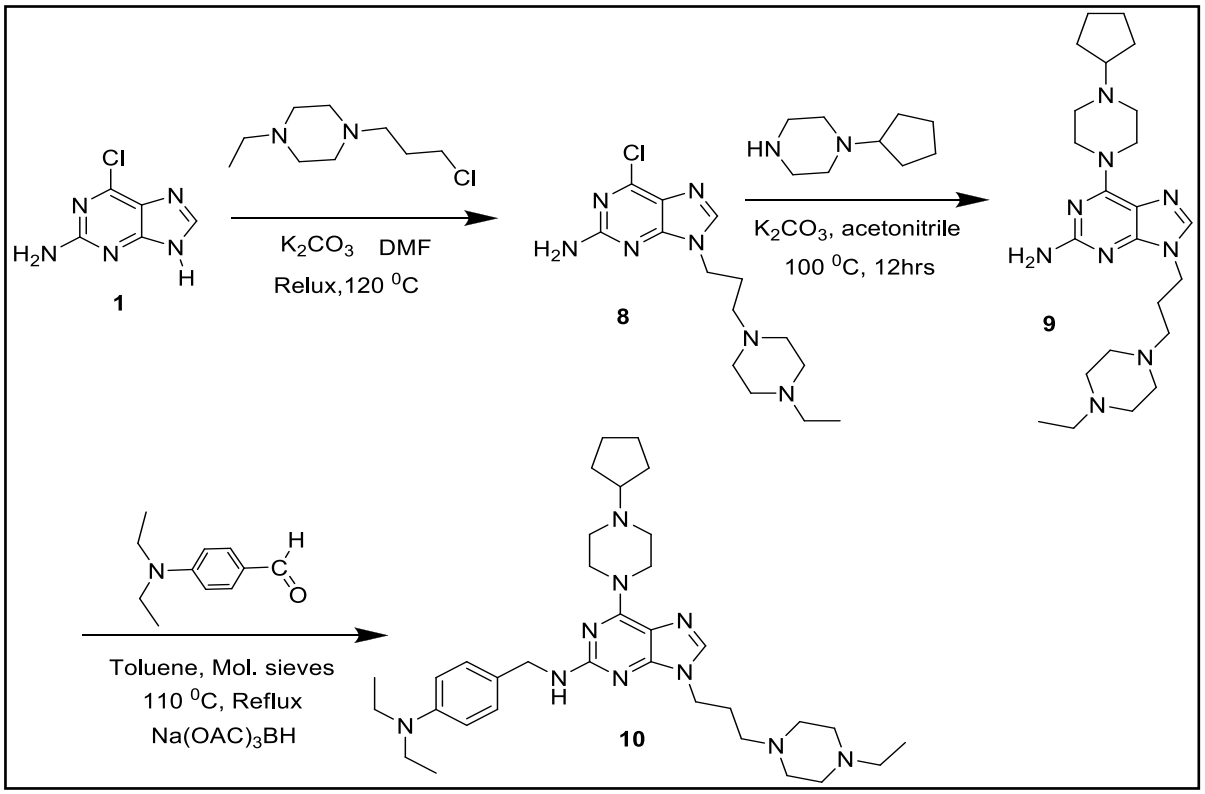
4.1 Scheme1:



Compound **2** was prepared by using 6-chloro-9H-purine and ethylpiperizine as starting materials and potassium carbonate as a base, in acetonitrile at reflux for 3-4h. Compound **2** was treated with 1-bromo-3-chloro propane in the presence of K_2CO_3 as a base, in DMF at room temperature for 12h to give compound **3**. Compound **3** was taken in a seal tube treated with the ethylpiperazine and potassium carbonate as a base, in DMSO at 100 ˚C for 24 hrs the residue was separated by the column chromatography to give compound **4**. Subsequently,

compound **4** was treated with the 4-methoxybenzaldehyde under refluxing condition in presence of the molecular sieves in toluene for 24 hours, thereafter toluene was evaporated and sodium triacetoxy borohydride was added and the reaction mixture was stirred for one hour, residue was separated by column chromatography to get compound **5.** Compound **4** was similarly treated with 4-fluorobenzaldehyde under refluxing condition in presence of molecular sieves in toluene for 24 hours. Subsequently sodium triacetoxy borohydride was introduced and the reaction stirred for one hour at room temperature, the residue was separated by the coloumn chromatography to get the compound **6.** Compound **4** was treated with 1,4-dibromobutane in DMF, NaH used as a base at 80 ^{o}C and stirred for 24 hours, residue was separated by the column chromatograph, to get compound **7**.

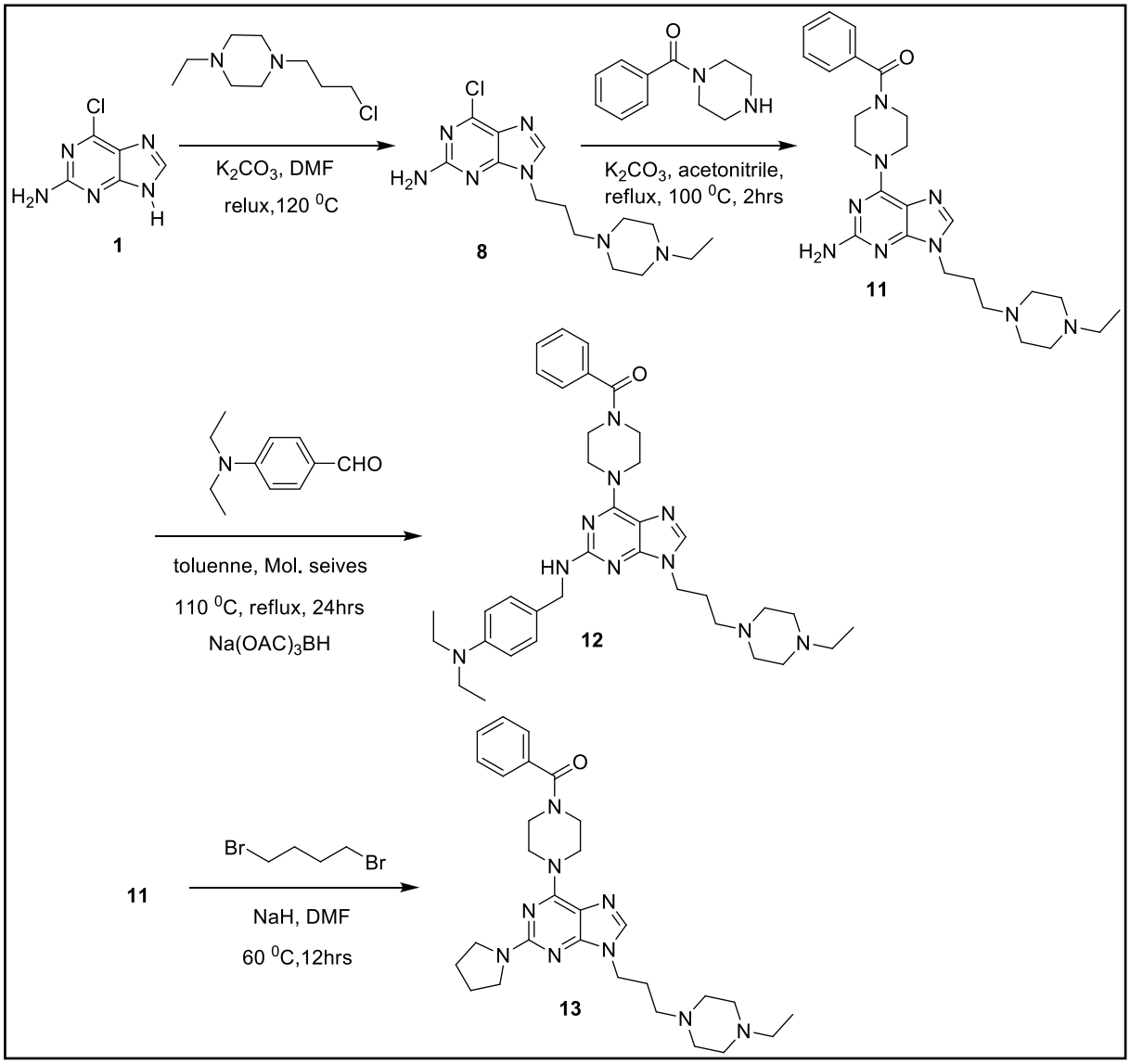
4.2. Scheme 2.



Compound **8** was prepared by the treatment of 6-chloro-9H-purine with the 1-(3-chioropropyl)-4-ethylpiperazine, potassium carbonate as a base in DMF at 120^{o}C for 24 hrs the residue was separated by the column chromatography. Compound **8** was treated with the

1-cyclopentenyl-4-ethylpiperazine, potassium carbonate as a base, in acetonitrile at 100 ^{0}C for 12 hours, residue was separated by the column chromatography to give compound **9.** Compound **10** was treated with 4-diethyl amino benzaldehyde in toluene at 110 ^{o}C for 24hrs, then after add the sodium triacetoxy borohydride, residue was separated by column Chromatography, to get the compound **10.**

4.3. Scheme 3.



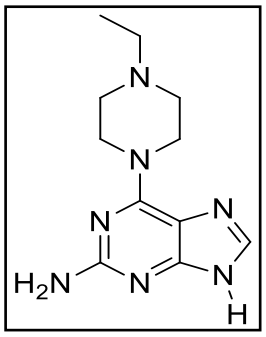
Compound **8** was prepared by treating compound **1** with 1-(3-choloropropyl)-4-ethylpiperazine in presence of potassium carbonate as a base in DMF at 120 ^{o}C for 24 hours.

The residue was separated column chromatography, to give compound **8**, which was treated with phenyl (piperazin-1-yl)methanone and potassium carbonate under refluxing condition, in acetonitrile for 12 hrs at 100 ^{o}C to give compound **11**. Thereafter, compound **11** was treated with 4-diethyl aminobenzaldehyde in toluene under refluxing condition for 24hrs. The solvent was evaporated and sodium triacetoxyborohydride was introduced and the reaction mixture stirred for 2 hours to give the compound **12**. Compound **11** was also treated with 1, 4-dibrobutane in DMF, at 60 ^{0}C for 12 hours, residue is separated by column chromatography to get the compound **13**.

5. Experimental data:

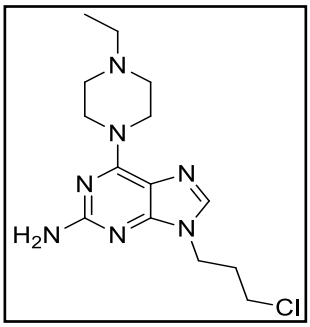
5.1. Scheme 1:

Synthesis of 6-(4-ethylpiperazine-1-yl)-9H-purin-2-amine (2): 1-Ethylpiperazine (1.01 g, 8.84 mmol) was added to a stirred suspension of 6-chloro-9H-purin-2-amine (1 g, 5.89 mmol) and potassium carbonate (0.8 g, 5.89 mmol) in dry acetonitrile. The mixture was heated at reflux for 3-4 hrs. Acetonitrile was removed under vacuum, the residue then washed with water, followed by filtration and dried to obtain compound **2** as a light brown solid (1.3 g, 89%). Melting point was found as > 250 ◦C. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.61 (s, 1H), 4.22-4.31 (m, 7H), 2.56-2.63 (m, 4H), 2.50 (q, *J* = 7.14 Hz, 2H), 1.15 (t, *J* = 7.23 Hz, 3H).



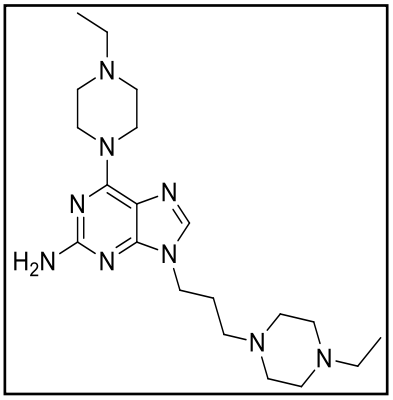
Compound **2**

Synthesis of 9-(3-chloropropyl)-6-(4-ethylpiperazine-1-yl)-9H-purin-2-amine(3): 1-Bromo-3-chloropropane (2.4 mL, 24.26 mmol) was added to a stirred suspension of compound **2** (2 g, 8.08 mmol) and potassium carbonate (1.1 g, 8.08 mmol) in dry DMF and the reaction mixture was stirred for 12 h at room temperature. Water was added to the reaction mixture. The organic solution was extracted with 20% methanol in chloroform and the aqueous layer was dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by column chromatography eluting with 5% methanol in chloroform, to give compound **3** (1.65 g, 63%) as gummy liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.50 (s, 1H), 4.61 - 4.71 (m, 2H), 4.18 - 4.36 (m, 6H), 3.50 (t, *J* = 6.13 Hz, 2H), 2.56 (t, *J* = 1.0 Hz, 4H), 2.46 (q, *J* = 7.14 Hz, 2H), 2.31 (q, *J* = 1.00 Hz, 2H), 1.13 (t, *J* = 7.23 Hz, 3H); ESIMS *m/z* (rel intensity) calculated: 323.28, found: 324.43 (MH+, 100).



Compound **3**

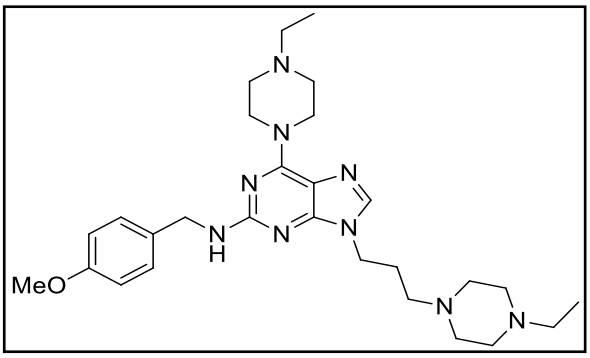
Synthesis of 6-(4-ethylpiperazin-1-yl)-9-(3-(4-ethylpiperazin-1-yl) propyl)-9H-purin-2-amine (4): A solution of compound **3** (1 g, 3.09 mmol) and triethylamine (1.29 mL, 9.28 mmol) dissolve in dry DMSO was taken in a seal tube. 1-Ethylpiperazine (0.43 mL, 3.4 mmol) was added to the stirred mixture. The mixture was heated at 100 °C for 12h. Water was added to the reaction mixture. The organic layer was extracted with 20% MeOH and chloroform system.. The aqueous layer was dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by column chromatography, by using chloroform and methanol system to get compound **4** (0.65 g, yield 53%) as red colour liquid. ^{1}HNMR (300 MHz, $CDCl_3$) δ ppm 7.48 (s, 1H), 4.61 (s, 2H), 4.16-4.32 (m, 4H), 4.07 (t, *J* = 6.75 Hz,2H), 2.54 (t, *J* = 5.03 Hz, 5H), 2.38-2.51 (m, 8H), 2.35 (t, *J* = 1.00 Hz, 1H), 2.30 (t, *J* = 6.90 Hz, 2H), 2.20 (m, 2H) 2.01-1.94 (m, *J* = 6.84 Hz, 2H), 1.11 (t, *J* = 7.19 Hz, 3H), 1.07 (t, *J* =7.23 Hz, 3H); ESIMS *m/z* (rel intensity) calculated: 401.55, found:402.47(MH^+,100) (MNa^+,35).



Compound **4**

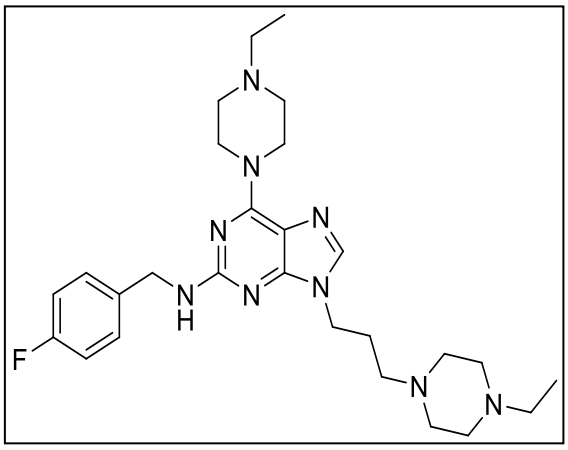
6-(4-ethylpiperazin-1-yl)-9-(-3-ethylpiperazin-1-yl)propyl)-N-(4-methoxybenzyl)-9H-purin-2-amine (5): Compound **4 (0.** 3g, 0.74 mmol) and 4-diethylaminobenzaldehyde (0.10

mL, 0.89 mmol) was dissolved in toluene (5 mL). One pinch of molecular sieves was added and the reaction stirred at 110 °C under N_2 atmosphere for 12-16 hrs. Thereafter toluene was evaporated and sodium triacetoxy borohydride (0.3 g, 1.48 mmoL) was added. The reaction was stirred at rt for 1-2 hrs. Reaction mixture was neutralized with $NaHCO_3$ solution. Organic part was extracted with 20% $CH_3OH/CHCl_3$ system. Column chromatography was done by using $CHCl_3$:CH_3OH system to get pure product of **5**, (0.06g, yield 30%) as a red colour liquid**.** ^{1}HNMR (300MHz, $CDCl_3$) δ ppm 7.27 (s, 3H) 6.5(d, *J* = 9 Hz, 2H), 4.97(s, 1H), 4.54 (d, *J* = 6 Hz, 2H), 4.24(s, 3H), 4.07(d, *J* = 6 Hz, 2H), 3.79 (s, 3H), 2.54(s, 5H), 2.42(t, *J* = 6 Hz, 5H), 2.30(d, *J* = 6 Hz, 3H), 1.98(t, *J* = 6 Hz, 2H), 1.13(s, 1H), 1.07(d, *J* =6 Hz, 6H) ESI-MS *m/z* (rel. intensity 100%) 522.60 $(M+H)^+$.



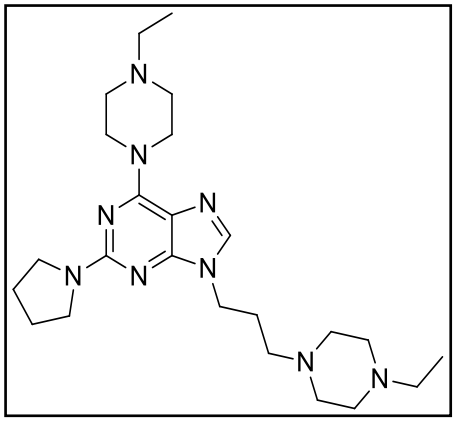
Compound **5**

Synthesis of 6-(4-ethyl piperazin-1-yl)-9(3-(4-ethylpiperazin-1-yl)propyl)-N-(4 Fluorobenzyl)-9H-purin-2-amine (6): Compound **4** (0.3g, 0.74 mmol) and 4-fluorobenzaldehyde (0.23g, 0.88 mmol) was dissolved in toluene(5 mL). One pinch of molecular sieves was added and the reaction stirred at 150 °C under N_2 atmosphere for 12-16 hrs. Thereafter toluene was evaporated and sodium triacetoxy borohydride (0.3g, 1.48) and DCE (5 mL) were added to the reaction mixture and subsequently stirred at rt for 1-2 hrs. Reaction mixture was neutralized with $NaHCO_3$ solution. Organic part was extracted with 20% $CH_3OH/CHCl_3$ system. Column chromatography was done by using $CHCl_3$:CH_3OH system to get pure product of **6** (0.05g, yield 30%) as a gummy liquid. ^{1}H NMR (300MHz, $CDCl_3$) δ ppm7.32(s, 2H) 6.98(m, *J* = 9 Hz, 2H), 4.57(d, *J* = 6 Hz, 1H), 4.22(s, 3H), 4.06(d, *J* = 6 Hz, 2H), 2.40-2.54(m, 11H), 2.25(d, *J* = 9 Hz, 2H), 1.94-199(m, 3H), 1.25(s, 2H), 1.08(t, *J* = 9 Hz 5H): ESIMS *m/z* (rel intensity 100%) 510.45 $(M+H)^+$.



Compound **6**

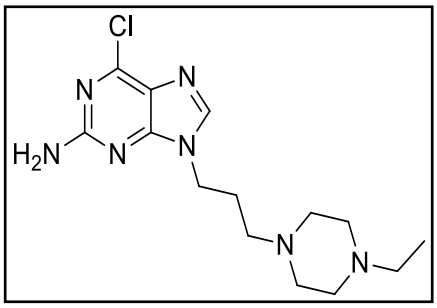
Synthesis of 6-(4-ethyl piperazin-1-yl)-9(3-(4-ethylpiperazin-1-yl)propyl-2-(pyrrolidin-1-yl)-9H-purine (7): Compound **4** (0.23g, 0.57 mmol) was dissolved in DMF (5 mL), cooled to 0 °C and NaH (0.12g, 0.85 mmol) and 1,4dibromobutane (1.5mL, 1.7 mmol) were added. The reaction was stirred under the N2 atmosphere condition at 80 °C for 24 hrs. Water was added to the reaction mixture and the organic layer was extracted by the 20% CH_3OH+$CHCl_3$ system. Column chromatography was done by using 20% CH_3OH+$CHCl_3$+NH_3 system to get the compound **7,** (0.04g, yield 30%) as a gummy liquid. **1**H NMR (300MHz, CDCl3) δ ppm 7.41(s, 1H), 4.24(s, 5H), 4.07(t, *J*=6 Hz, 3H), 3.5(t, *J*=6 Hz, 5H), 2.42-2.46(q, 11H), 2.30(d, *J*=9 Hz, 5H), 1.89-1.94 (m, 7H), 1.06-1.13(m, 8H). ESIMS *m/z* (rel intensity 100%) 456.48 (M+H)+.



Compound **7**

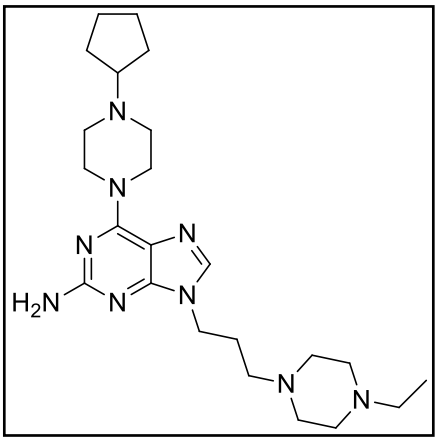
5.2 Scheme 2.

Synthesis of 6-chloro 9-(3-(4-ethyl piperazin-1-yl)propyl)-9H-purin-2-amine (8): Compound **8** (2g, 0.01 mmol) was dissolved in DMF(5 mL), and stirred for 2-3 hours under the N2 atmosphere condition at the 120 °C get the clear solution. Potassium carbonate and (1.6g, 0.1 mmol) ,(3-chloropropyl)-4-ethylpiperazine) were added to the reaction mixture and stirred for 12hours. Water was added to the reaction and organic layer was separated by the 20% $CH_3OH/CHCl_3$ system. Column chromatography was done by using $CHCl_3$+CH_3OH+NH_3 system, to get the compound **8, (**1.2gm, 50% yield) as a thick gummy liquid. ^{1}H NMR (300MHz, $CDCl_3$) δ ppm 7.81(s, 1H), 5.21(s, 2H), 4.14-4.19(t, 2H), 2.43(t, 10H) , 2.29(d, *J* = 6 Hz, 3H), 2.2(t, *J*=6 Hz, 3H) 1.08(t , 4H). ESIMS *m/z* (rel intensity 100%) 324.26 $(M+H)^+$.



Compound **8**

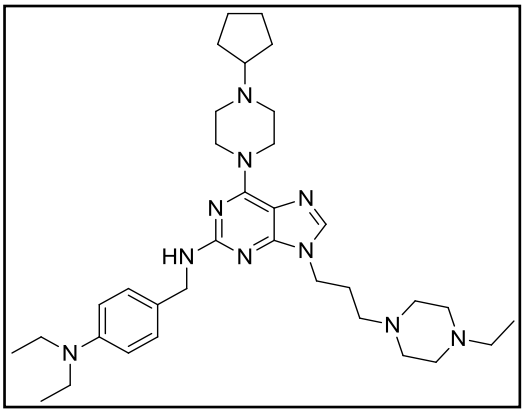
Synthesis of 6-(4-cyclopentylpiperazin-1-yl)-9-(3-(4-ethyl piperazin-1-yl)propyl)-9H-purin-2-amine (9): Compound **9** (0.1g, 0.36 mmol) was dissolved in acetonitrile (5 mL) and potassium carbonate (0.04g, 0.36 mmol) and 1-cyclopentylpiperazine (0.05g, 0.37 mmol)) were added. The reaction was stirred under the N2 atmospheric condition at 100 °C for 12hrs. Organic layer was extracted by the 20% $CH_3OH/CHCl_3$ system and column chromatography was done by using CH_3OH and $CHCl_3$ system to separate compound **9**, (0.02 g, yield 40%) as a brown liquid. ^{1}H NMR (300MHz, $CDCl_3$) δ ppm 7.46 (s, 1H), 4.71 (s, 2H), 4-4.10 (m, 5H), 2.24-2.56 (m, 18H), 1.93 (t, *J* = 6 Hz, 6H), 1-1.05(t, *J*=6 Hz, 5H). ESIMS *m/z* (rel intensity 100%) 442.45$(M+H)^+$.



Compound **9**

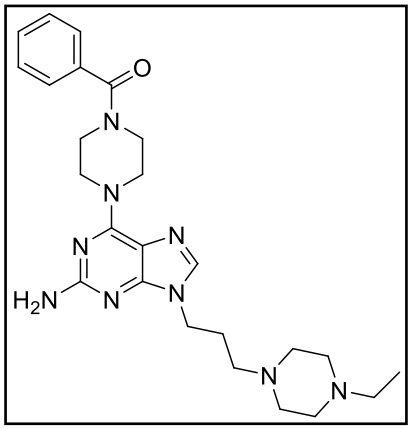
Synthesis of 6-(4-cyclopentyl piperazin-1-yl)-N-(4-diethylamino)benzyl)-9-(3-(4-ethylpiperazine-1-yl)propyl)-9H-purine-2-amine(10): Compound **9**(0.25g, 0.34mmol) and 4-diethyl aminobenzaldehyde (0.20g, 0.60 mmol) was dissolved in toluene (5 mL). One pinch of molecular sieves was added and the reaction stirred at 110 °C under N_2 atmosphere for 12-16 hrs. Thereafter toluene was evaporated and sodium triacetoxy borohydride (0.2g, 0.68 mmol) was added to the reaction mixture and stirred for 2-3 hrs at rt. Reaction mixture was neutralized with $NaHCO_3$ solution. Organic part was extracted with 20% $CH_3OH/CHCl_3$ system. Column chromatography was done by using $CHCl_3$:CH_3OH system to get pure product of **10, (**0.02g**.** yield 25%) as a gummy liquid. ^{1}H NMR (300MHz, $CDCl_3$) δ ppm 7.43 (s, 1H), 7.19 (d, *J* = 9.0 Hz, 2H), 6.61 (d, *J* = 9 Hz, 2H), 4.45 (d, *J* = 3 Hz, 2H), 4.42 (s, 3H) 4.06 (t, *J* = 6. Hz, 2H), 3.27-3.34 (m, 3H), 2.50-2.57 (m, 5H), 2.37-2.42(m, 9H), 2.28,(d, *J*=6 Hz, 3H), 1.97 (t, *J* = 6. Hz, 2H), 1.86 (s, 2H), 1.68 (d, *J* = 6. Hz, 2H), 1.41-155 (m, 4H), 1.03-(m, 10H). ESIMS *m/z* (rel intensity 100%) 603.64 $(M+H)^+$.

Compound **10**



5.3 Scheme 3.

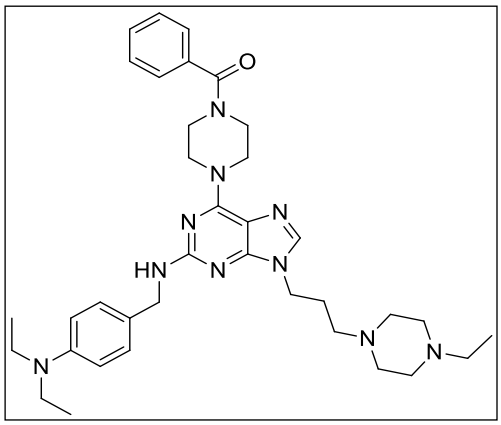
Synthesis of (4-(2-amino-9-(3-(4-ethylpiperazin-1-yl)propyl)-9H-purin-6-yl)piperazin-1-yl)(phenyl)methanone (11). Compound **8** was dissolved in acetonitrile (5 mL), potassium carbonate (1.2g, 0.03 mmol) and phenyl(piperazine-1-yl) methanone (0.84g, 0.03 mmol) were added. The reaction was stirred for 2 hours under N2 atmosphere condition at 100 °C. Acetonitrile was evaporated under the vacuum, then water was added to the reaction mixture to get precipitate which was filter off to get precipitate (0.8g, yield 70%) of product 11. No need to do Column Chromatography for this step. ^{1}H NMR (300MHz, $CDCl_3$) δ ppm 7.49 (s, 1H), 7.42 (d, *J* = 9 Hz, 4H), 4.63 (s, 2H), 4.26 (s, 3H), 4.09 (t, *J* = 6 Hz, 2H), 3.89 (s, 2H), 3.54 (s, 2H), 2.42 (d, *J* = 6 Hz, 8H), 2.28-2.40 (m, 3H), 1.85-2.03 (m, 3H), 1.08 (t, *J* = 6 Hz, 3H). ESI-MS *m/z* (rel intensity 100%) 478.34 $(M+H)^+$



Compound **11**

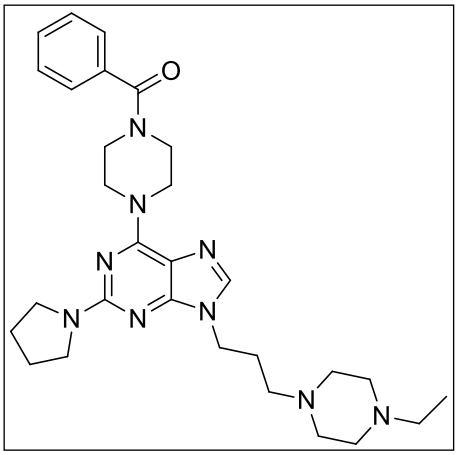
Synthesis of 4-(2-((4-(diethylamino)benzyl)amino-9-(3-(4-ethylpiperazin-1-yl) propyl)-9H-purine-6-yl)piperazin-1-yl)(phenyl)methanone(12). Compound **11** (0.2g, 0.41 mmol) and 4-diethyl aminobenzaldehyde (0.07g, 0.41mmol) was dissolved in toluene (5 mL). One pinch of molecular sieves was added and the reaction stirred at 150 °C under N_2 atmosphere for 12-16 hrs. Thereafter toluene was evaporated and sodium triacetoxy borohydride (0.18g, 0.82 mmol) to the reaction mixture and the mixture was dissolved in DCE (5 mL). Reaction mixture was kept in room temperature and allowed to stirred for 2-3 hours. After completion of the reaction, it was neutralized with $NaHCO_3$ solution. Organic part was extracted with 20% $CH_3OH/CHCl_3$ system. Column Chromatography was done by using CHCl3:CH3OH system to get pure product of **12**, **(**0.06g, yield 30%) as a gummy liquid. **¹**H NMR (300 MHz, $CDCl_3$) δ ppm 7.47 (s, 6H), 7.19 (s, 2H), 6.63 (d, *J* = 9Hz, 2H), 4.96 (s, 1H), 4.45 (d, *J* =9 Hz, 2H), 4.21-4.27 (m, 3H), 4.07-4.12 (m, 2H), 3.87 (s, 2H), 3.29-3.36 (m, 4H), 2.44-2.49 (m,

9H), 2.32(d, *J* = 6 Hz, 4H), 1.98 (d, *J* = 6 Hz, 3H), 1.25 (s, 2H), 1.10-1.16 (m, 9H). ESIMS *m/z* (rel intensity 100%) 639.74 $(M+H)^+$.



Compound **12**

Synthesis of (4-(9-(3-(4-ethylpiperazin-1-yl)propyl)-2-(pyrrolidin-1-yl)-9H-purin-6-yl)piperazin-1-yl)(phenyl)methanone(13): Compound **11** (0.25g, 0.52 mmol) was dissolved in dry DMF (5 mL), the reaction mixture was cooled to -10 °C and NaH (0.02g 1.4 mmol) was added. The reaction mixture was allowed to stir for one hour at cold condition. Then 1,4-dibromo butane (0.06g, 0.52 mmol) was added to it and allowed to stir for further 12 hours under the N_2 atmosphere at 60 °C. After completion of the reaction, organic layer was extracted by using the $CHCl_3$/ CH_3OH system. Column chromatography was done by using $CHCl_3$/CH_3OH system to get pure product **13,** (0.02g, yield 30%) as a red colour gummy liquid. ^1H NMR (300MHz, $CDCl_3$) δ ppm 7.46 (s,1H), 6.98 (t, *J* = 9Hz, 3H), 4.57 (d, *J* = 6Hz, 1H), 4.22 (s, 2H), 4.07 (s, 2H), 2.40-2.54 (m, 11H), 2.28 (d, *J* = 6 Hz, 2H), 1.97 (d, *J* = 9 Hz, 3H), 1.25 (s, 2H), 1.08 (t, *J* = 9 Hz, 5H). ESIMS *m/z* (rel intensity 100%) 532.57$(M+H)^+$.

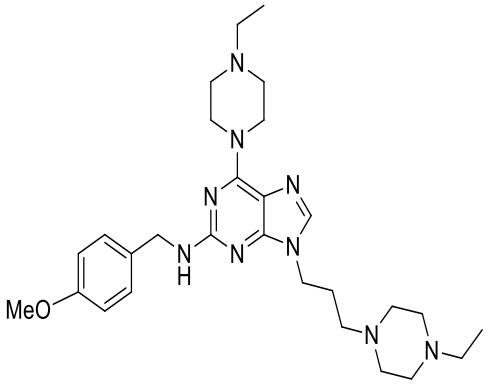
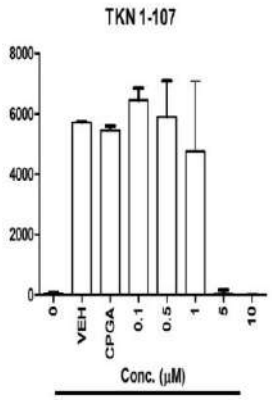
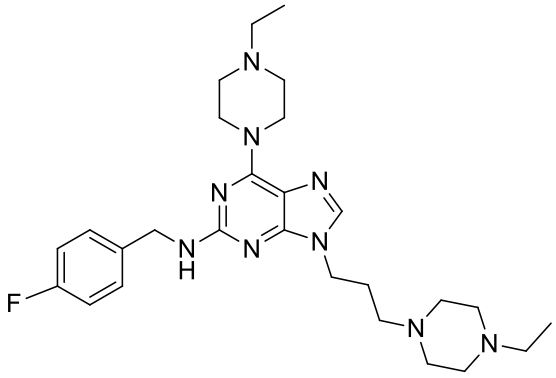
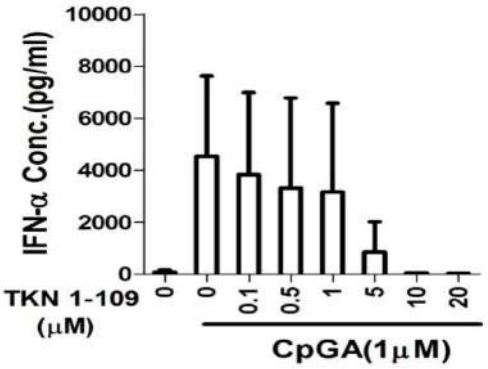


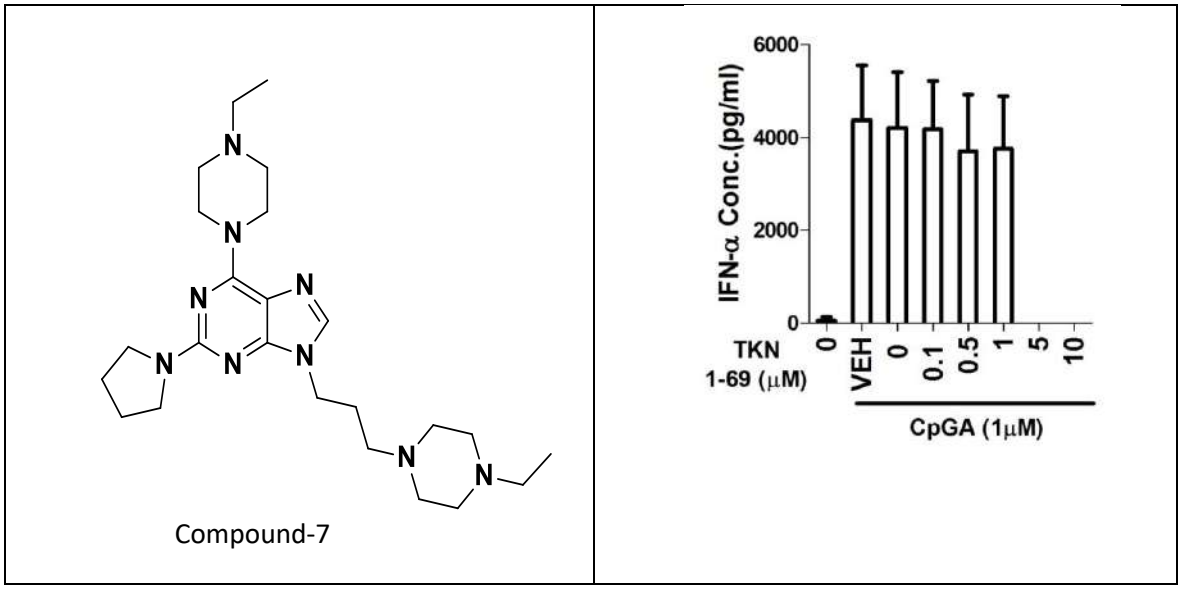
Compound **-13**

6. RESULTS AND DISCUSSION.

We have synthesised library of molecules, which were biologically evaluated by using Assay and ELISA. Some of these molecules have shown significant activity at 5 μM Concentration by inhibiting the IFN-α release. This indicates these molecules are having TLR-9 antagonist property, ranging from 5-10 μM.

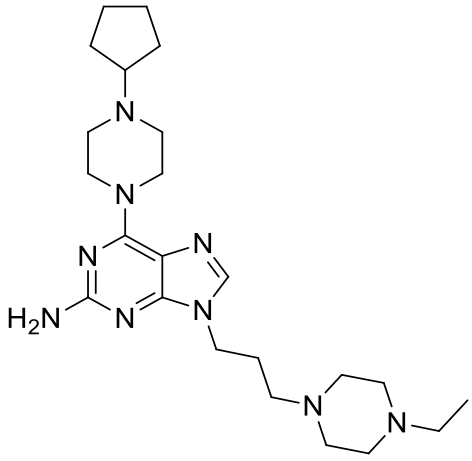
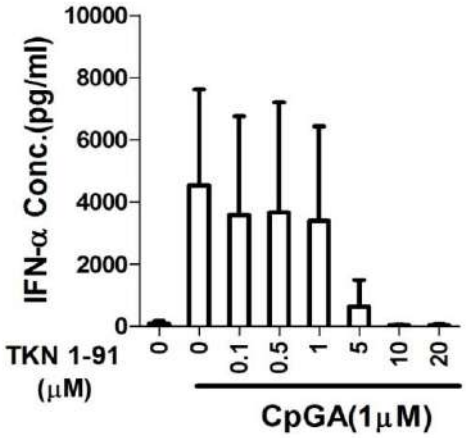
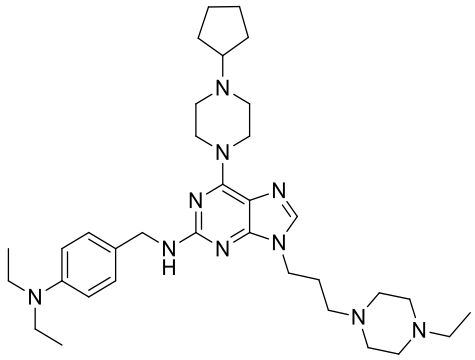
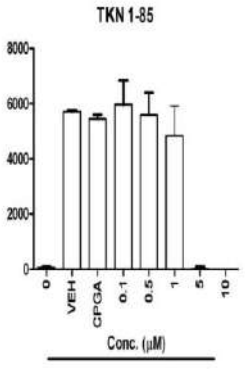
6.1. Scheme 1

Compounds	Activity
 Compound-5	
 Compound-6	

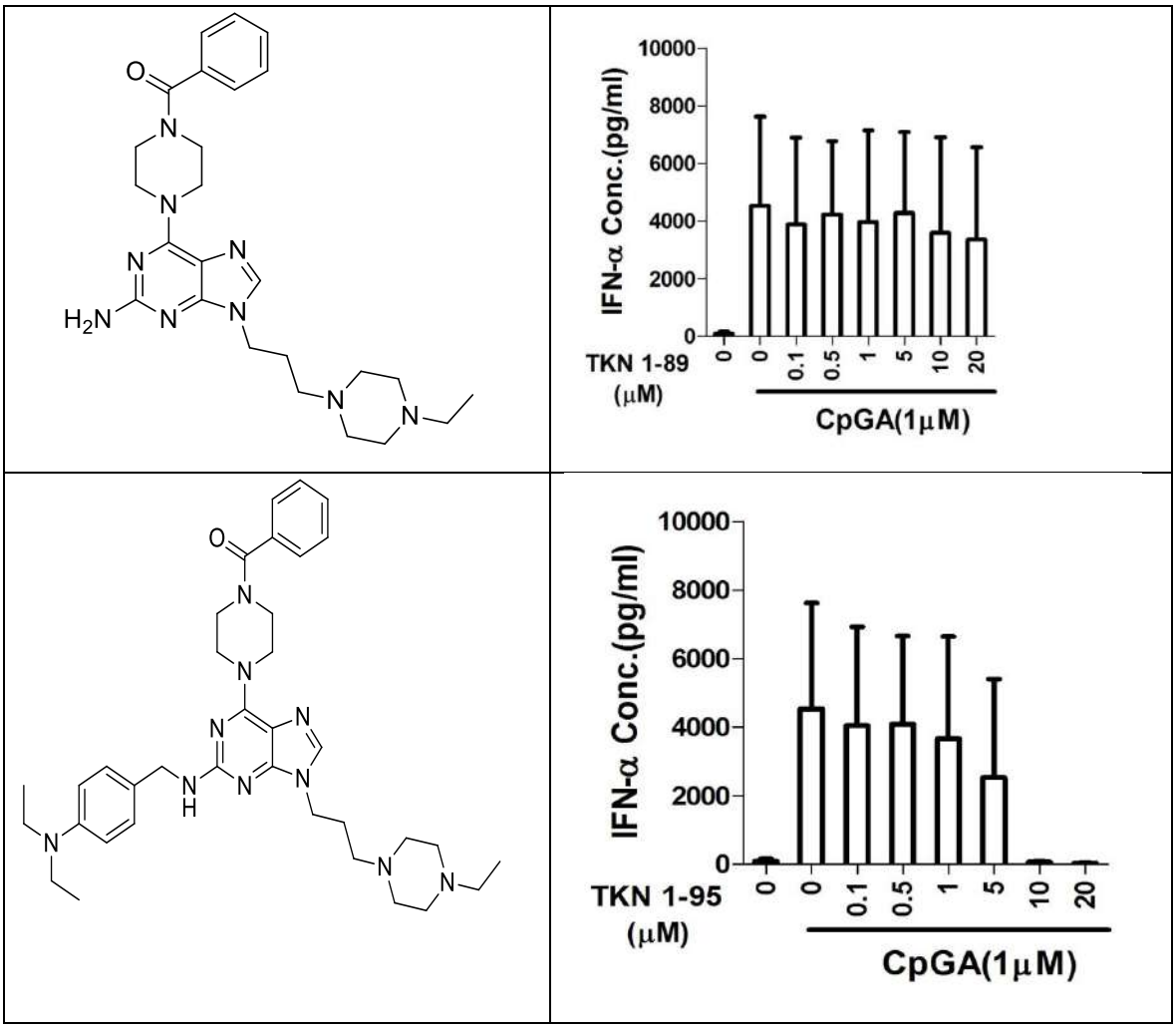


In this series of compound we have change the 4 position of purine ring. Introduction of methoxy group (Compound) and fluro group (compound) with hydrophobic phenyl ring does not change the activity profile. We have also attached pyrrolidine ring there which shows same trade. SAR study of this series revealed that change of such modification of donor acceptor propriety does not impact much in the activity all compound IC_{50} near 1 □M. In the next scheme 2 series we have modified in the substitution pattern of 6 position piperizene part.

6.2 <u>Scheme 2</u>. 6-Chloro-9H-purin-2-amine is the starting for compound8, compound 9, compound10, compound 11 were synthesised using the same strategy described in previous experimental section. The data obtained from the primary assay and ELISA for compounds 10 and 11 shown following results.

Compound	Activity
	
	

6.3 Scheme 3. Compounds which were prepared in scheme 1 and 2, are showing almost same results.So we prepared some other molecules starting from the same starting material which was shown in scheme 1 and scheme 2. But some modifications had been made on 6th position. The primary ELISAassay for the compounds 15 and 16 are showing the following results.



When we add benzoyl ring activity drastically decrease which implies that protonation of piperrizene amine is very much needed for activity. We benzoyl group is added it decrease the basicity of Nitrogen so activity shows proportionally decrease of activity.

7. Conclusion: We have synthesized disubstituted and trisubstituted purine analogues. Disubstituted purine analogues were failed to inhibit TLR9. We identified trisubstituted purine scaffold as a novel TLR9 inhibitor, which inhibits the release of IFN-α. Compound 5, 6, **9,** and compound **10** are inhibiting TLR9 at 5-10µM concentration. From the whole series of we conclude that balance between hydrophobicity and protonation of purine derivative is very essential for the activity which is eventually connect to physiological parameter logP.

A further SAR study has to be done to explain the importance of substitution positions. In the future we will focus on mechanism of action of these novel TLR9 inhibitors. Also we initiate ligand based mechanistic inside of TLR 9 antagonism using SAR study. For further modification we need to calculate IC_{50} value using reporter cell line.

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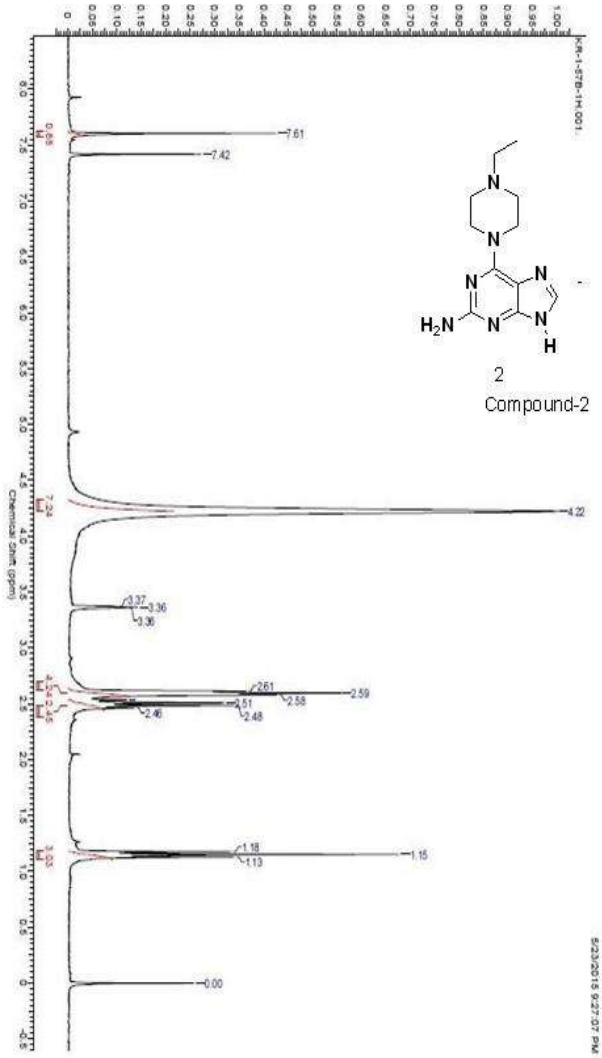
Supporting information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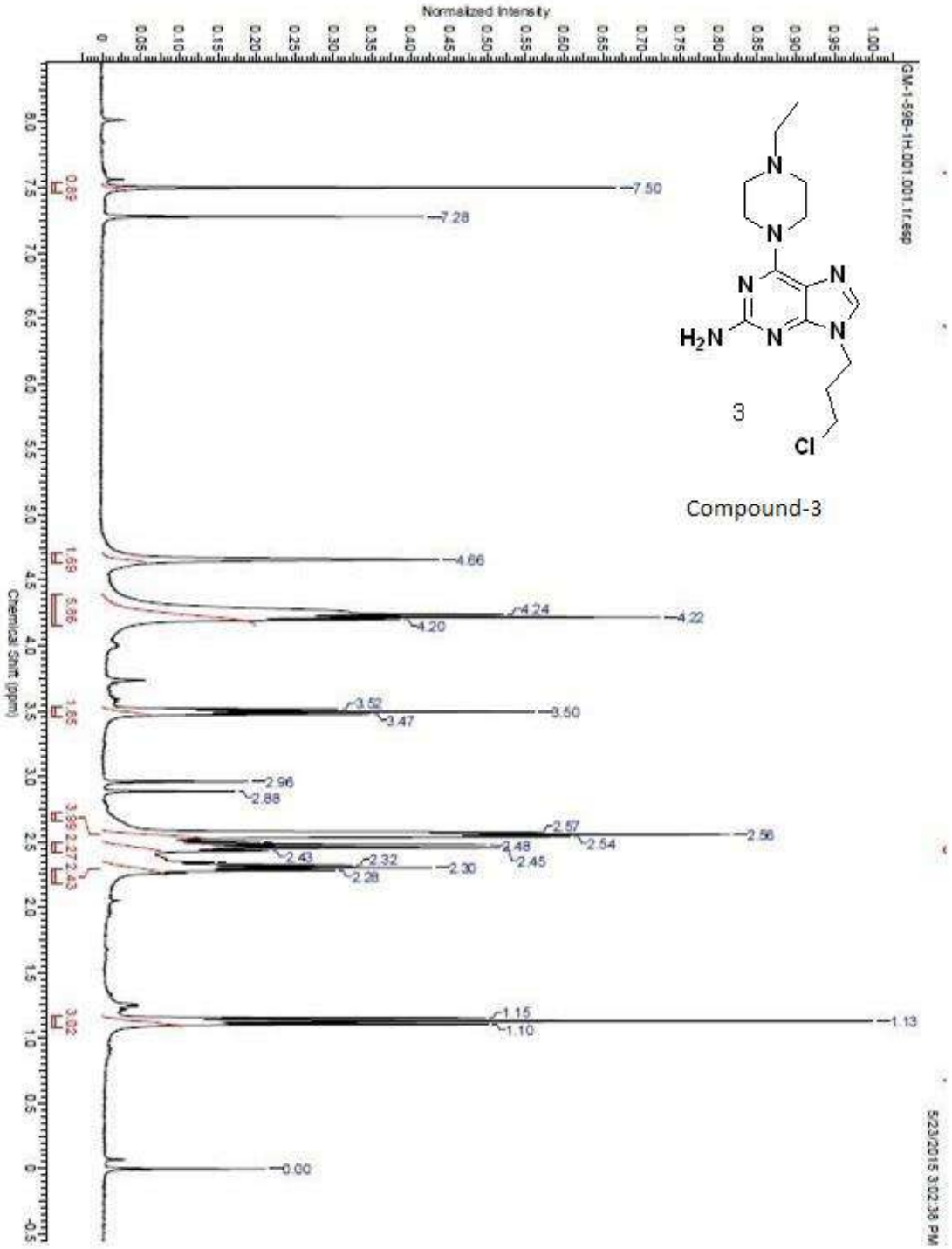
2.46
1.18
1.13
1.15
0.00
2
Compound-2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2.56
2.54
2.48
2.45
2.43
2.32
2.30
2.28
1.15
1.13
1.10
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0.89
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5.86
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3.99
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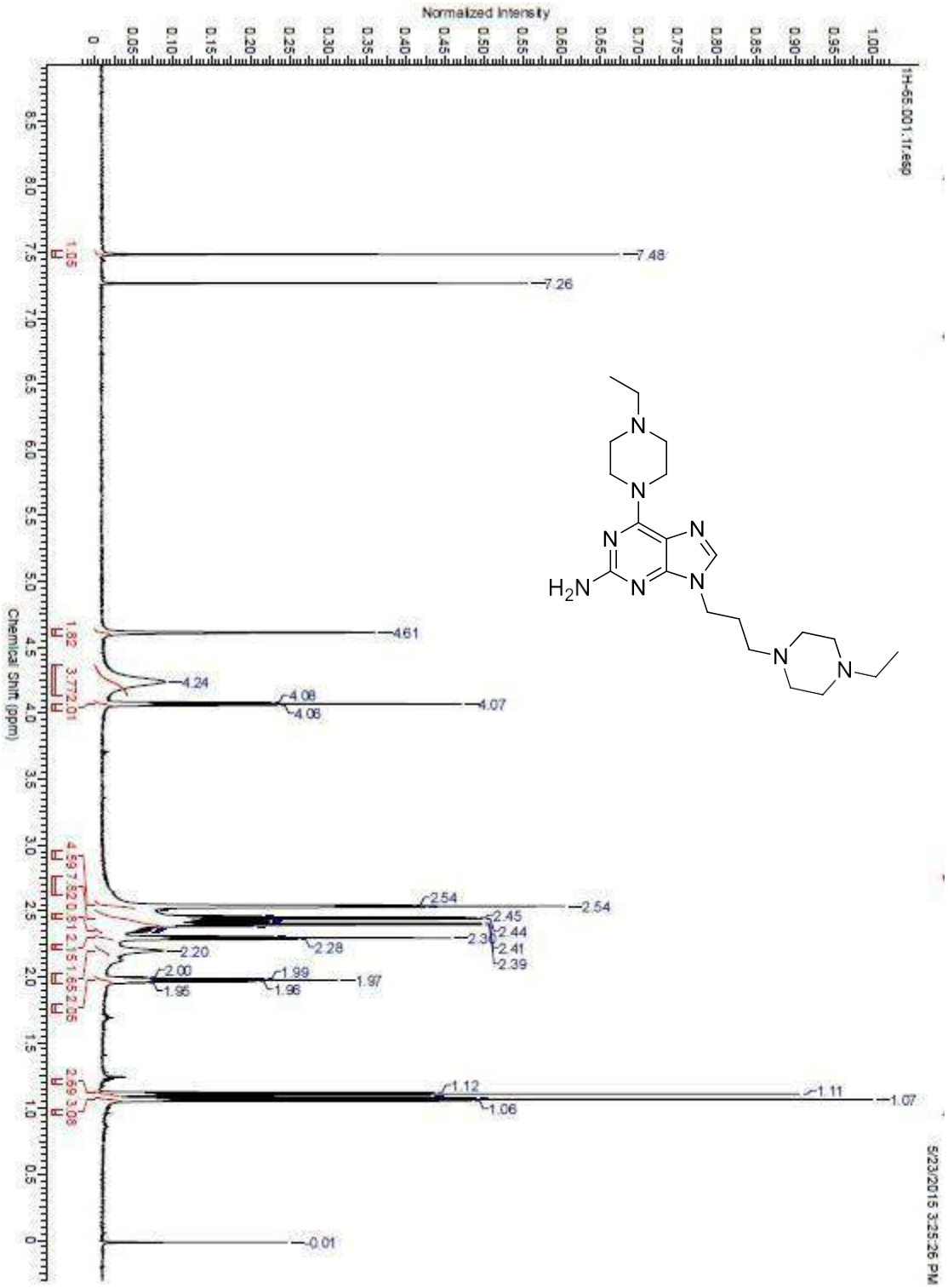
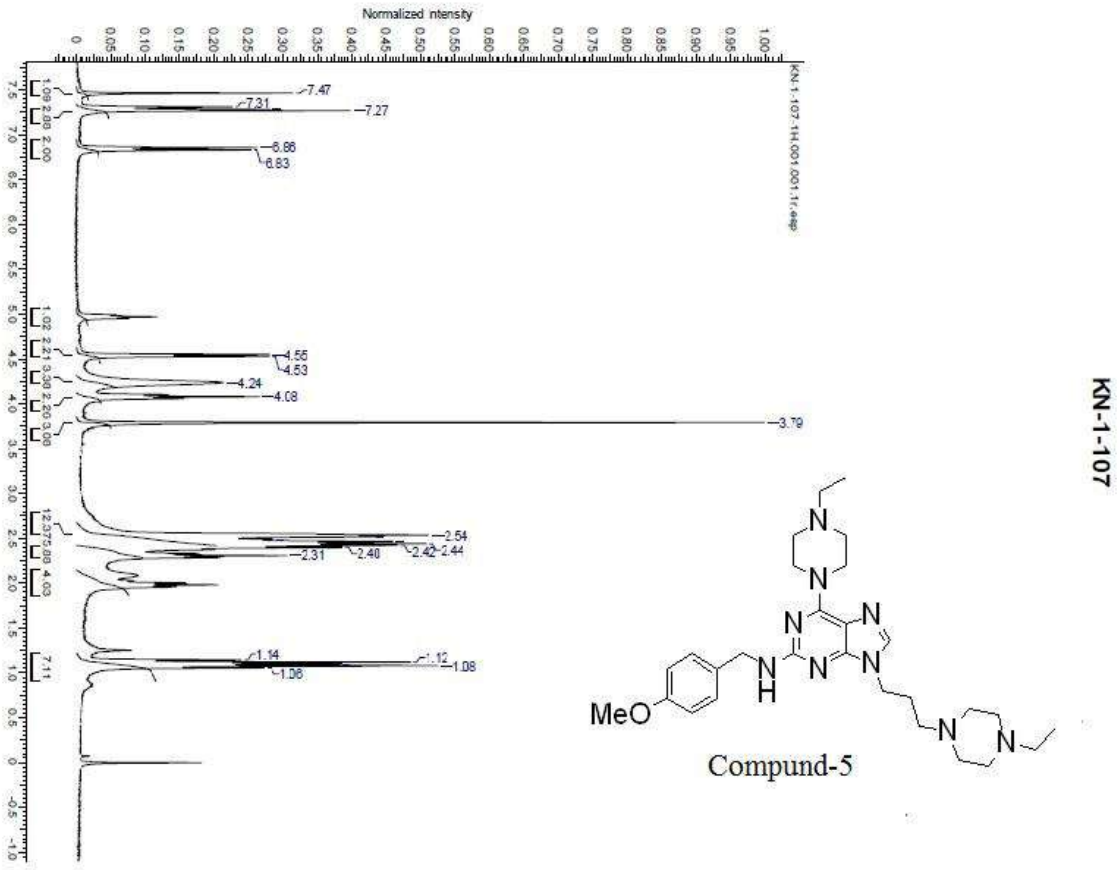
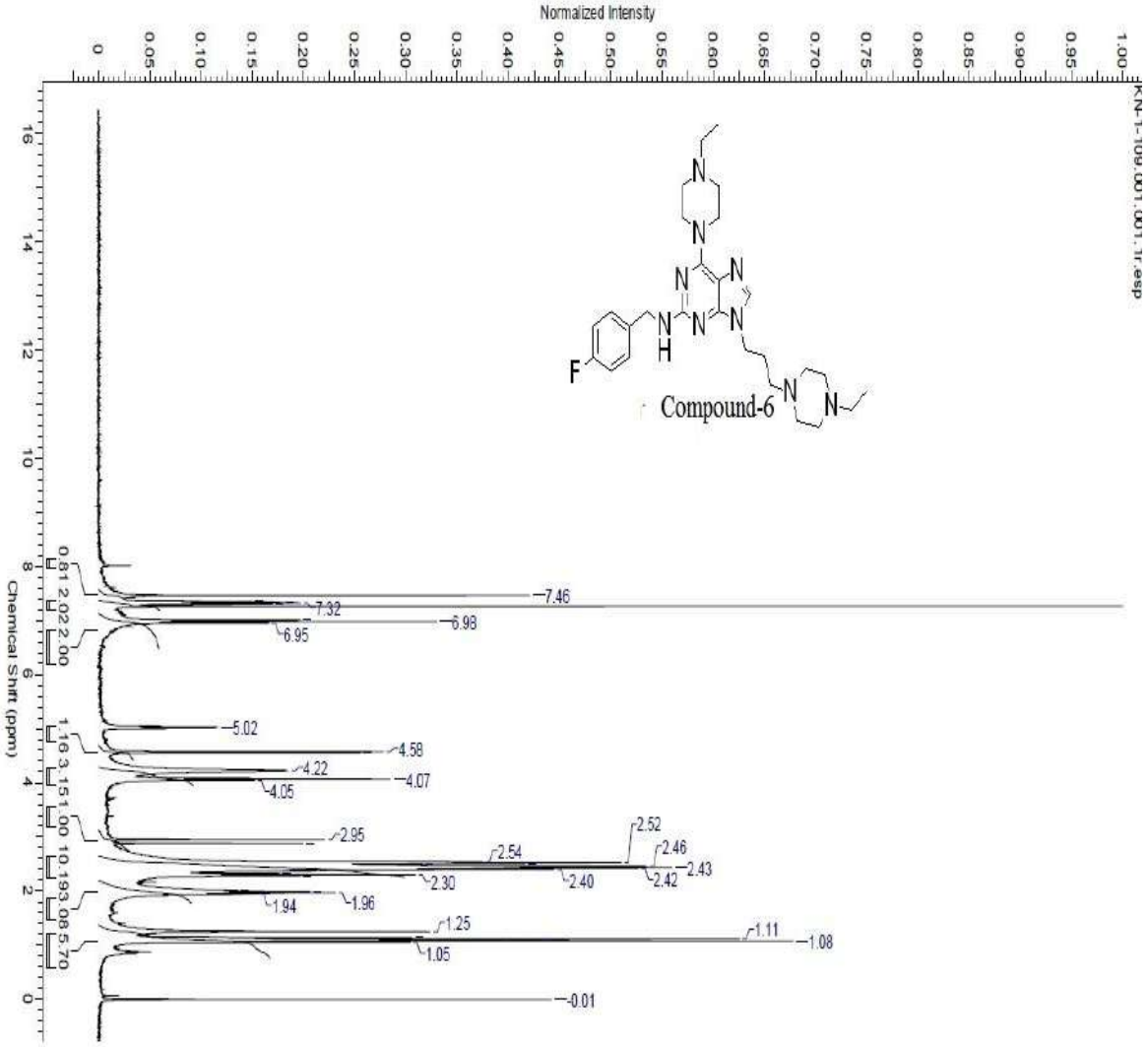
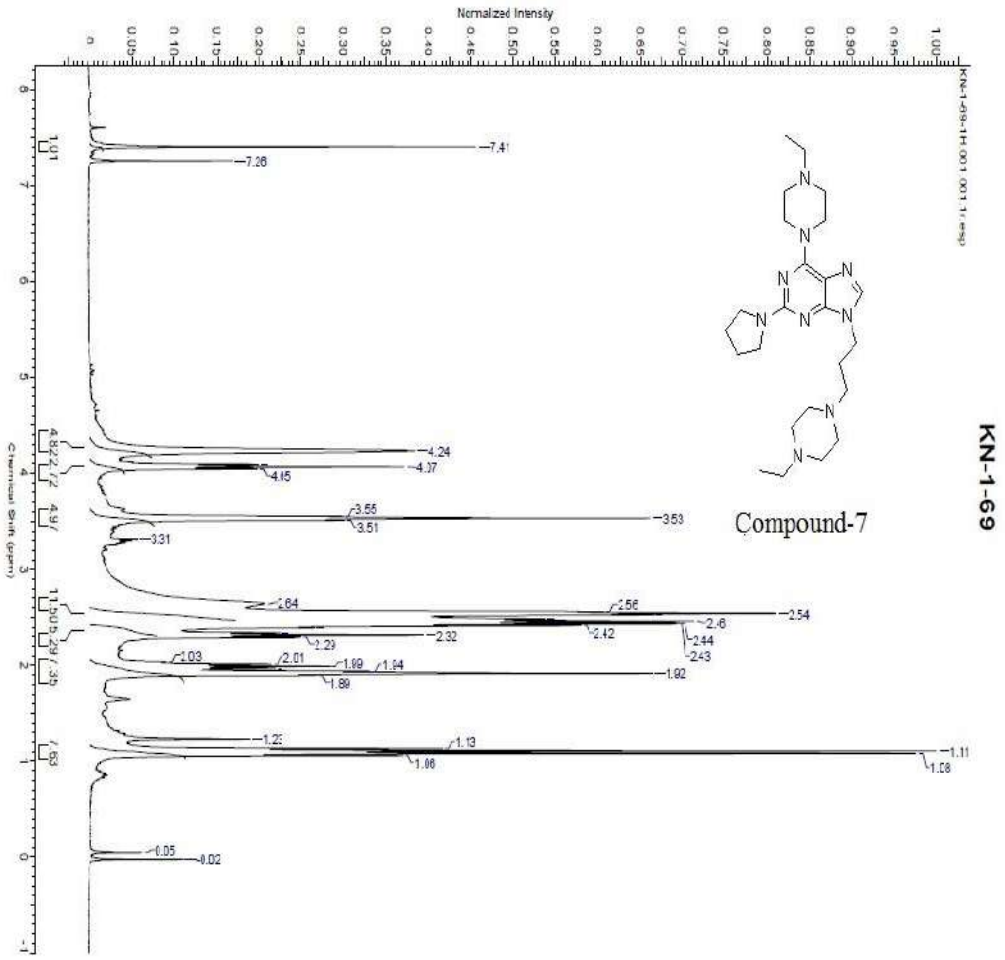
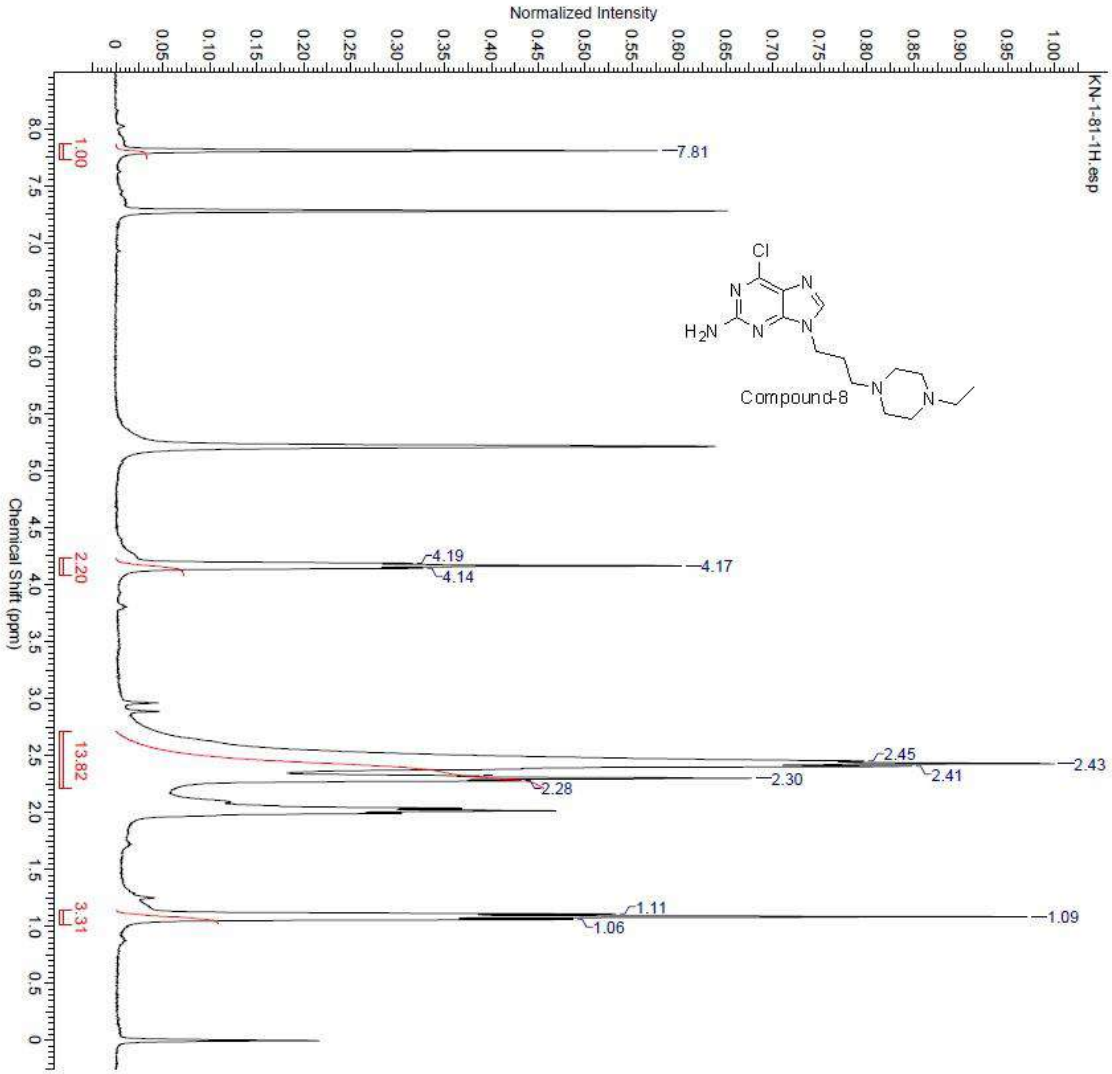
Cl
3
Compound-3

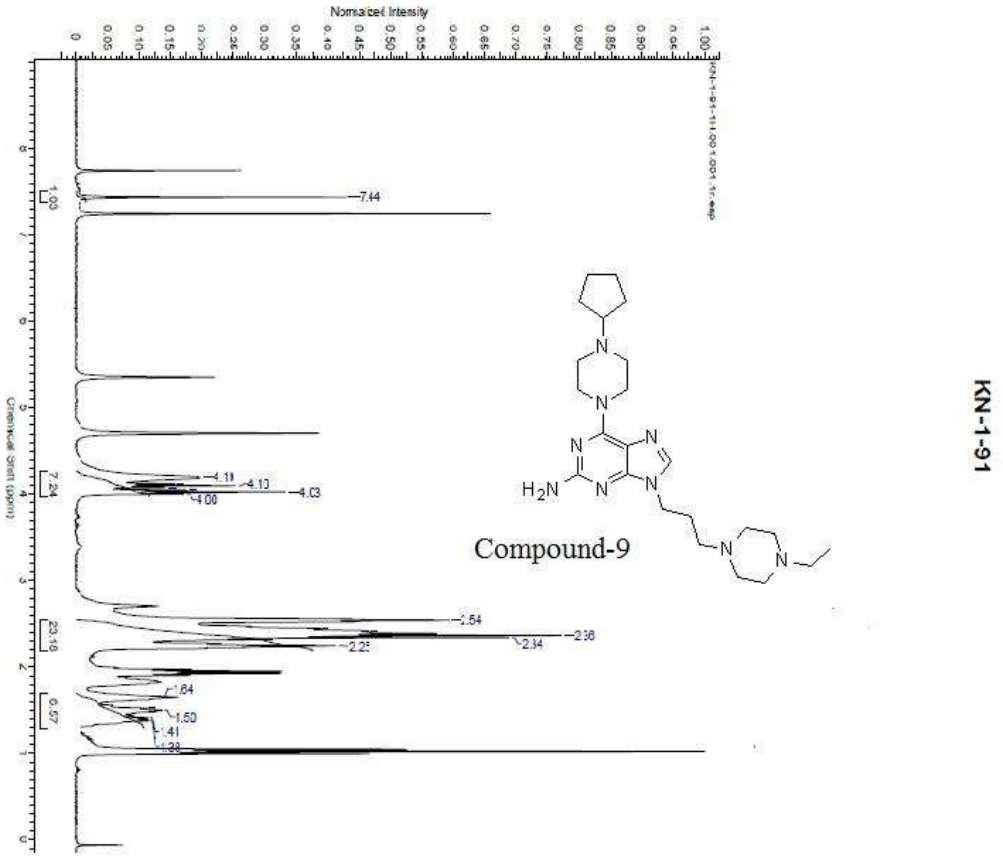

Normalized Intensity
Chemical Shift (ppm)
7.48
7.26
4.61
4.24
4.08
4.06
4.07
2.54
2.54
2.45
2.44
2.41
2.39
2.36
2.28
2.20
2.00
1.99
1.97
1.96
1.96
1.12
1.11
1.07
1.06
0.01
1H-55.001.1r.esp
5/23/2015 3:25:26 PM

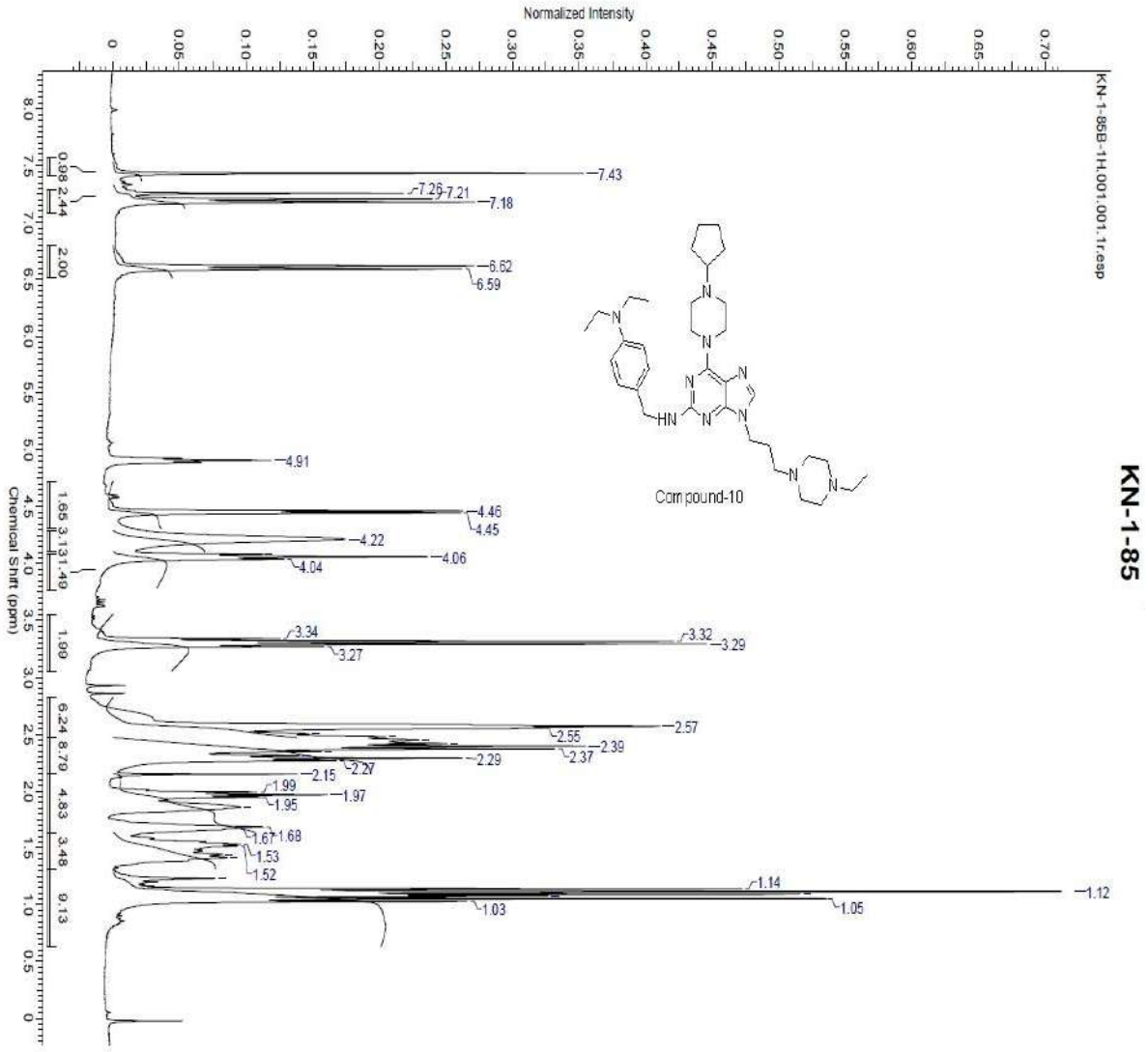

Normalized ntensity
KN-1-107
MeO
Compund-5

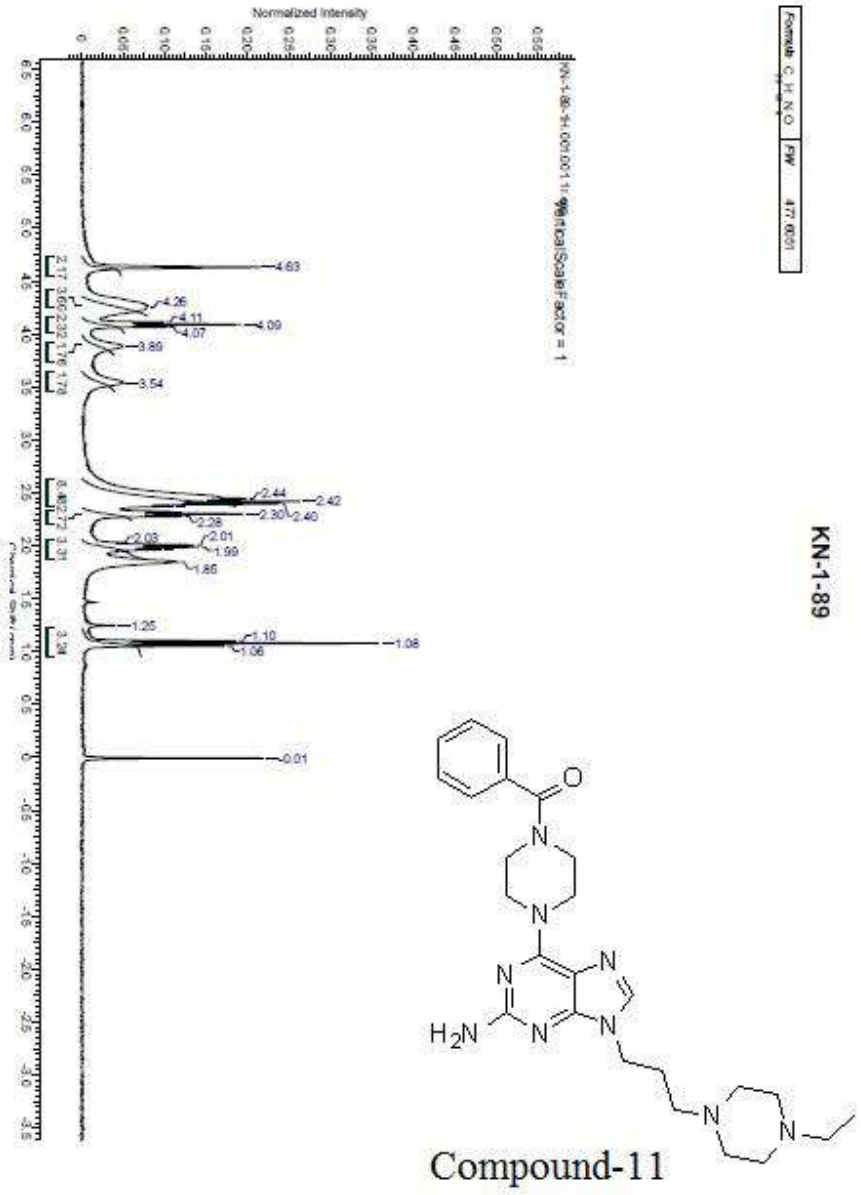

Normalized Intensity
Chemical Shift (ppm)
Compound-6
F
N
H
7.46
7.32
6.98
6.95
5.02
4.58
4.22
4.07
4.05
2.95
2.54
2.52
2.46
2.43
2.42
2.40
2.30
1.96
1.94
1.25
1.11
1.08
1.05
-0.01
0.81
2.02
2.00
1.16
3.15
1.00
10.19
3.08
5.70

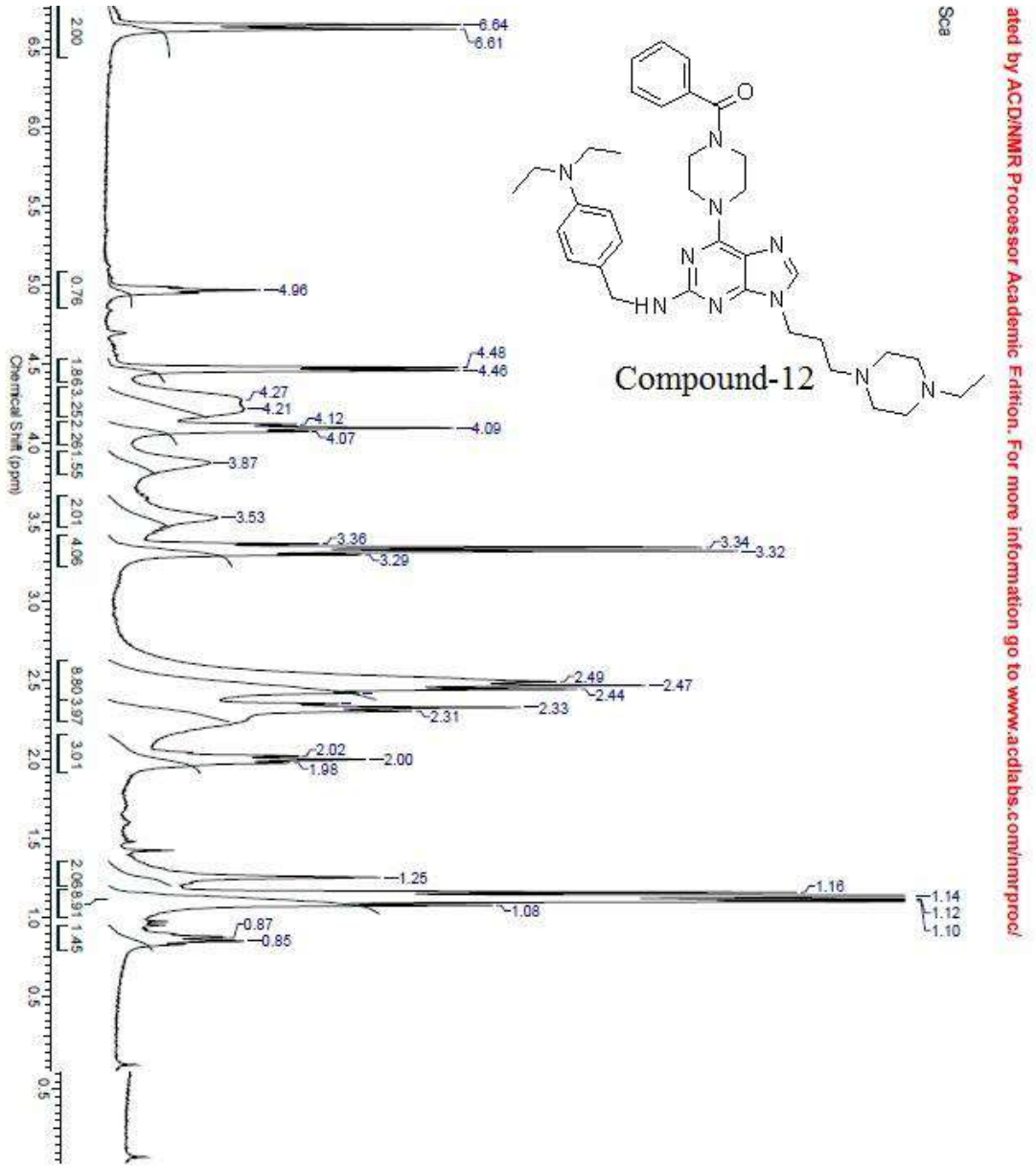

Normalized Intensity
Chemical Shift (ppm)
KN-1-69
Compound-7

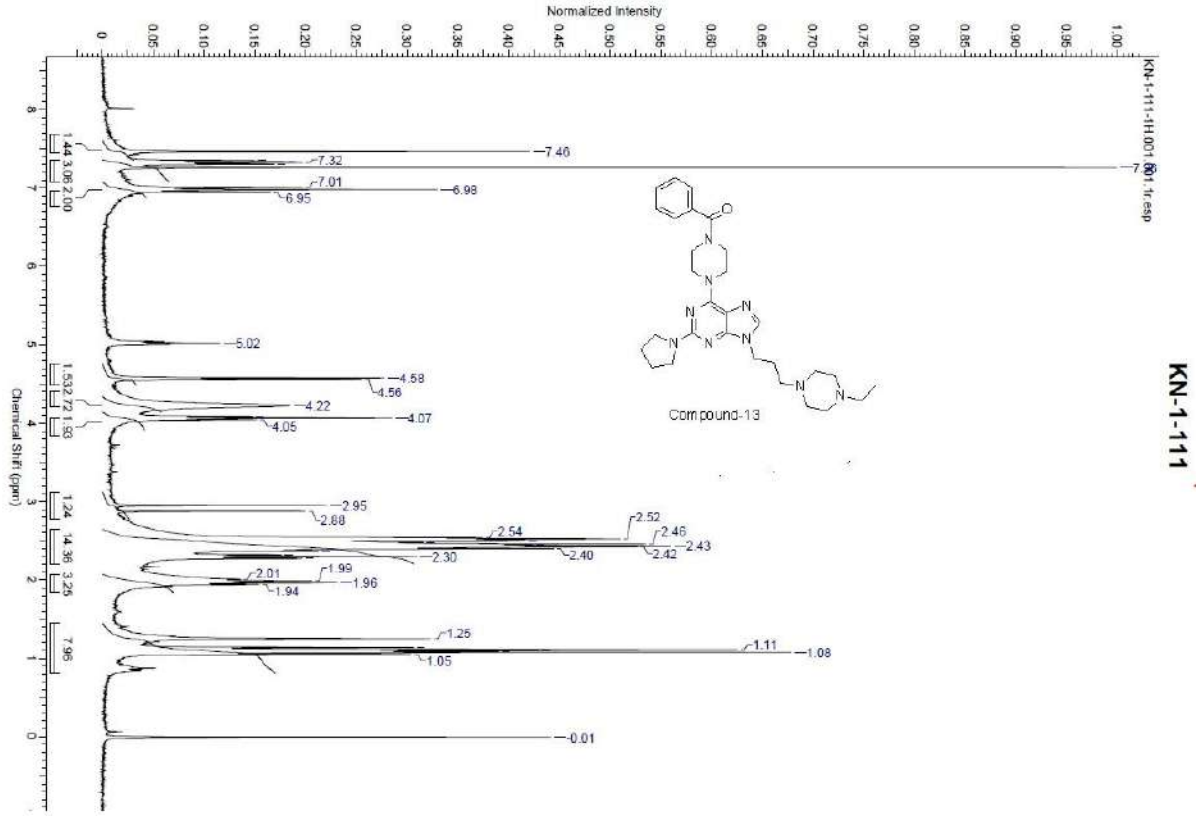

Normalized Intensity
KN-1-81-1H.esp
Chemical Shift (ppm)
Cl
N
N
N
N
H_2N
N
N
Compound-8
7.81
4.19
4.14
4.17
2.45
2.41
2.43
2.30
2.28
1.11
1.06
1.09
1.00
2.20
13.82
3.31

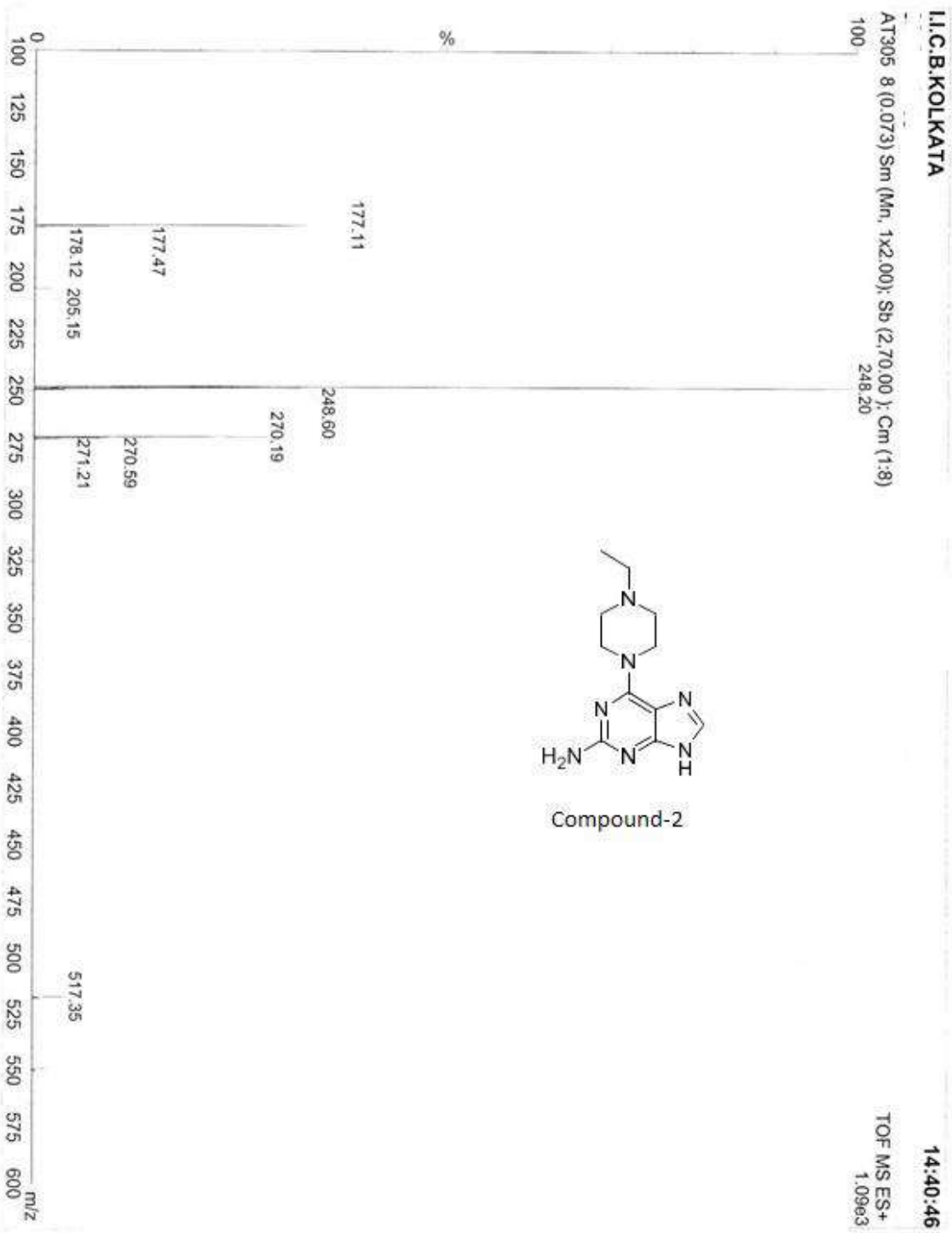

Normalized Intensity
Chemical Shift (ppm)
7.44
4.11
4.10
4.03
4.00
2.54
2.36
2.34
1.64
1.50
1.41
1.39
1.00
7.24
23.16
6.57
Compound-9
KN-1-91

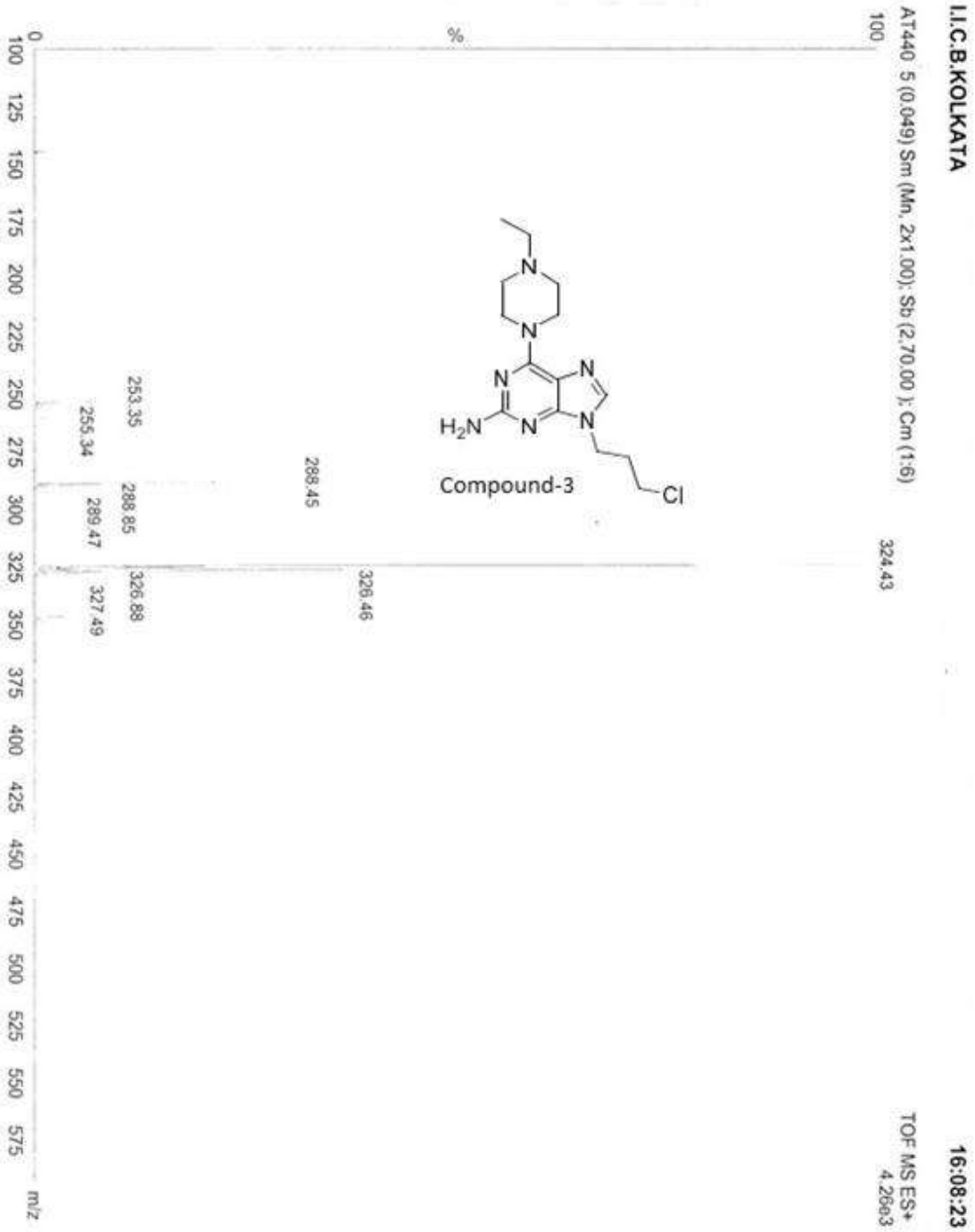

Normalized Intensity
KN-1-85B-1H.001.001.1r.esp
KN-1-85
Compound-10
Chemical Shift (ppm)
7.43
7.26
7.21
7.18
6.62
6.59
4.91
4.46
4.45
4.22
4.06
4.04
3.34
3.32
3.29
3.27
2.57
2.55
2.39
2.37
2.29
2.27
2.15
1.99
1.97
1.95
1.68
1.67
1.53
1.52
1.14
1.12
1.05
1.03
0.98
2.44
2.00
1.65
3.13
1.49
1.99
6.24
8.79
4.83
3.48
9.13

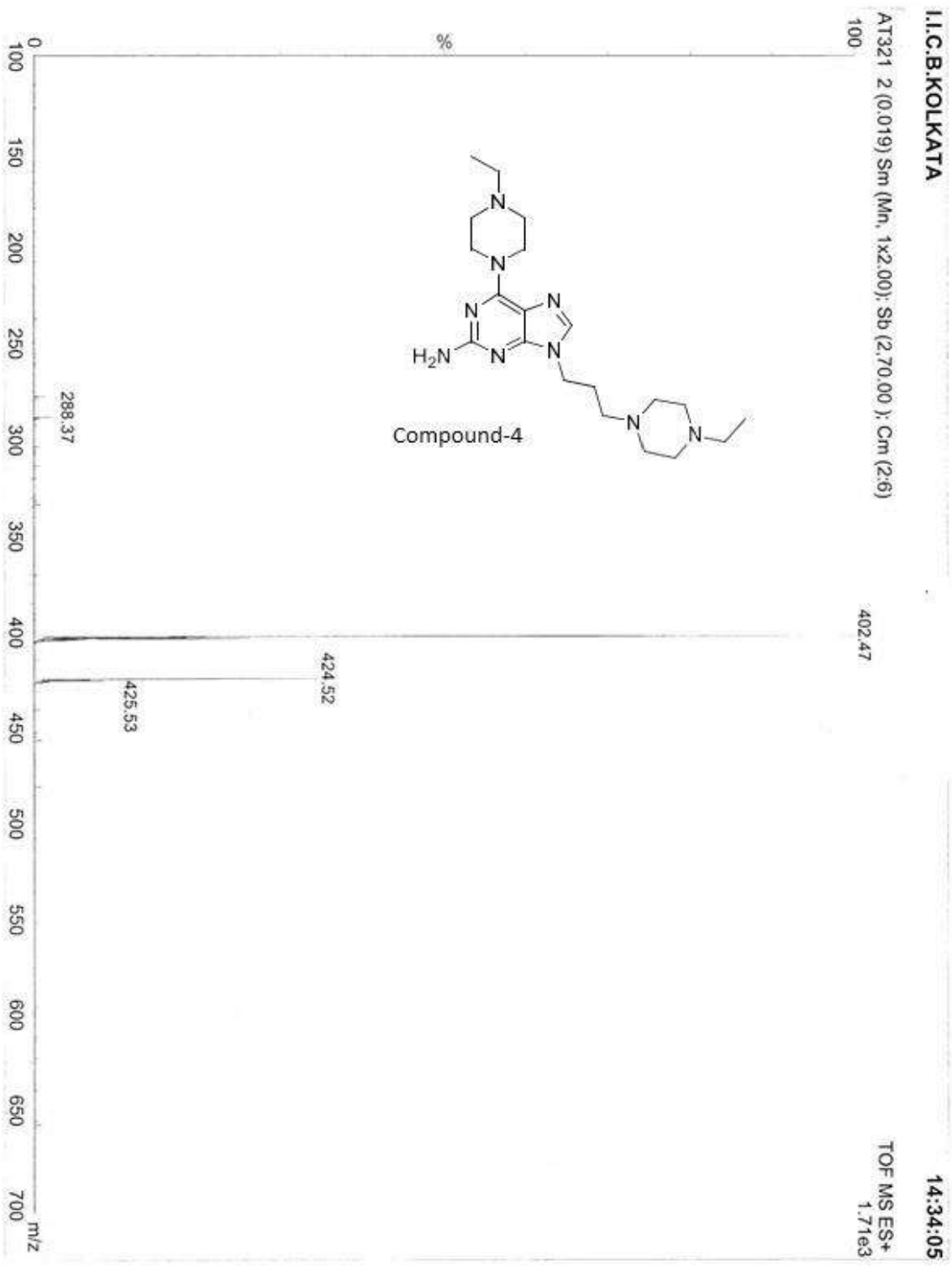

Normalized Intensity
KN-1-89
Compound-11

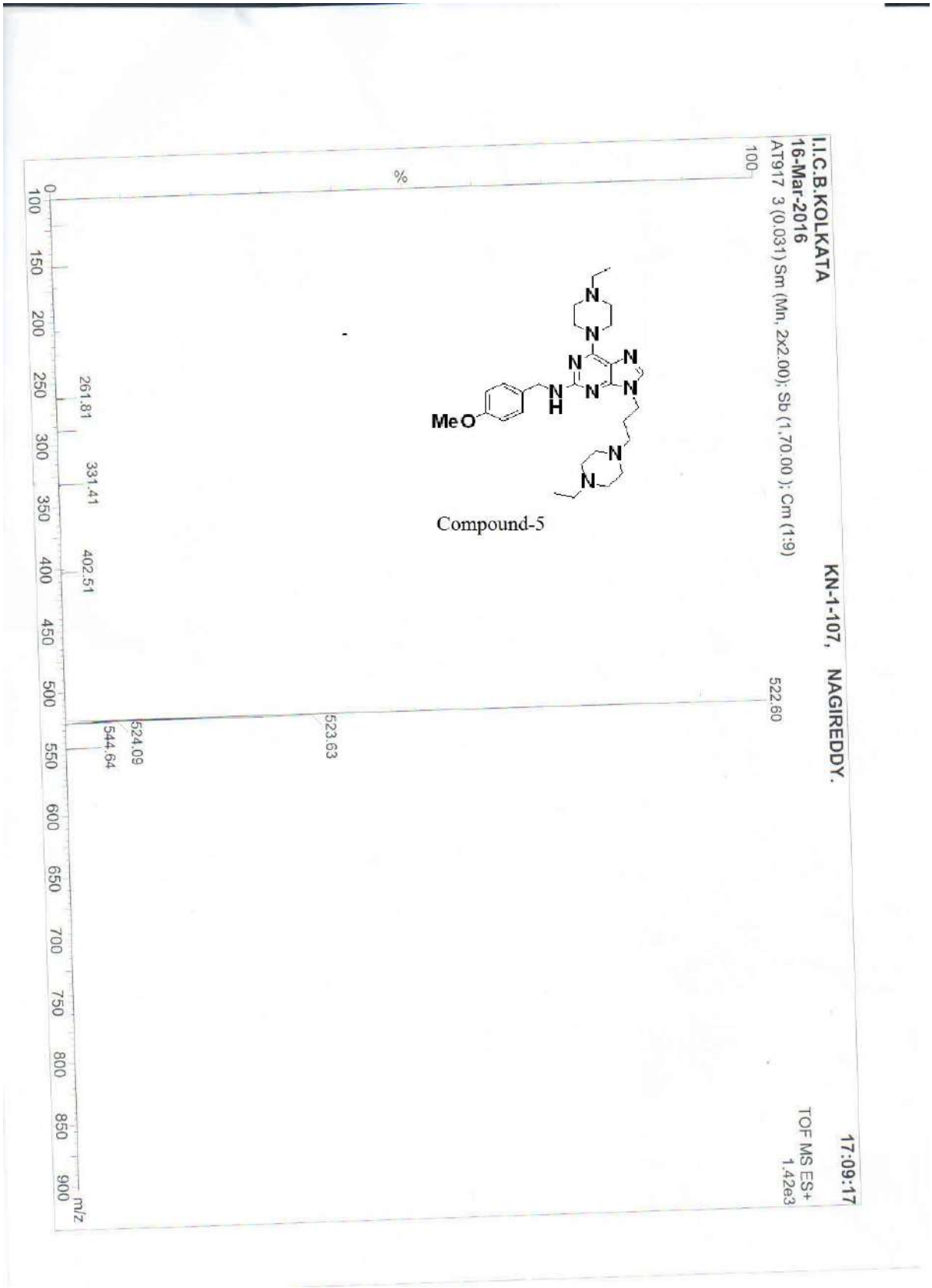

Compound-12
Chemical Shift (ppm)
ated by ACD/NMR Processor Academic Edition. For more information go to www.acdlabs.com/nmrproc/

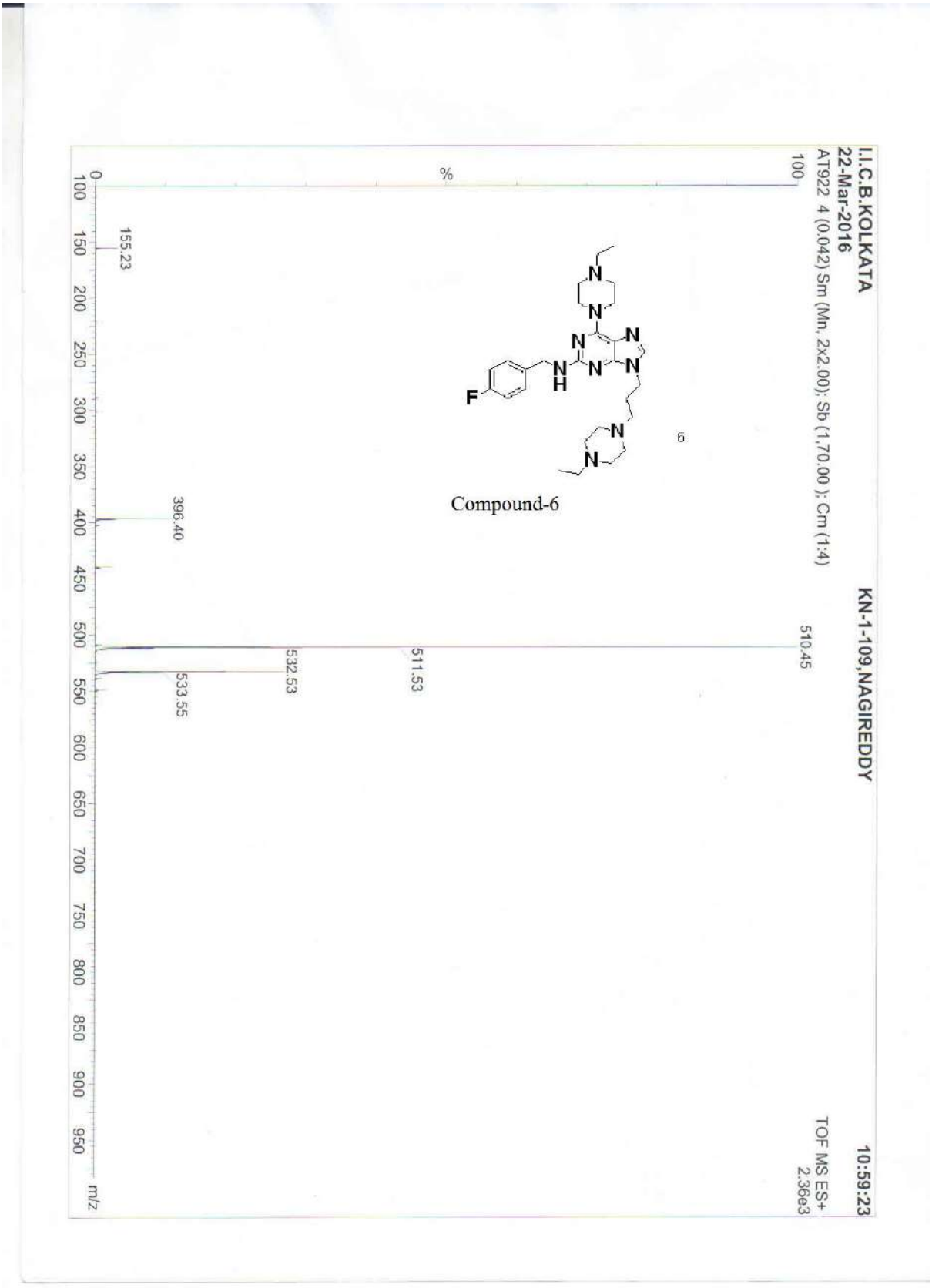

Normalized Intensity
Chemical Shift (ppm)
KN-1-111-1H-001-001.1r.esp
Compound-13
KN-1-111
7.46
7.32
7.01
6.98
6.95
5.02
4.58
4.56
4.22
4.07
4.05
2.95
2.88
2.54
2.52
2.46
2.43
2.42
2.40
2.30
2.01
1.99
1.96
1.94
1.25
1.11
1.08
1.05
0.01
1.44
3.06
2.00
1.53
2.72
1.93
1.24
14.36
3.25
7.96

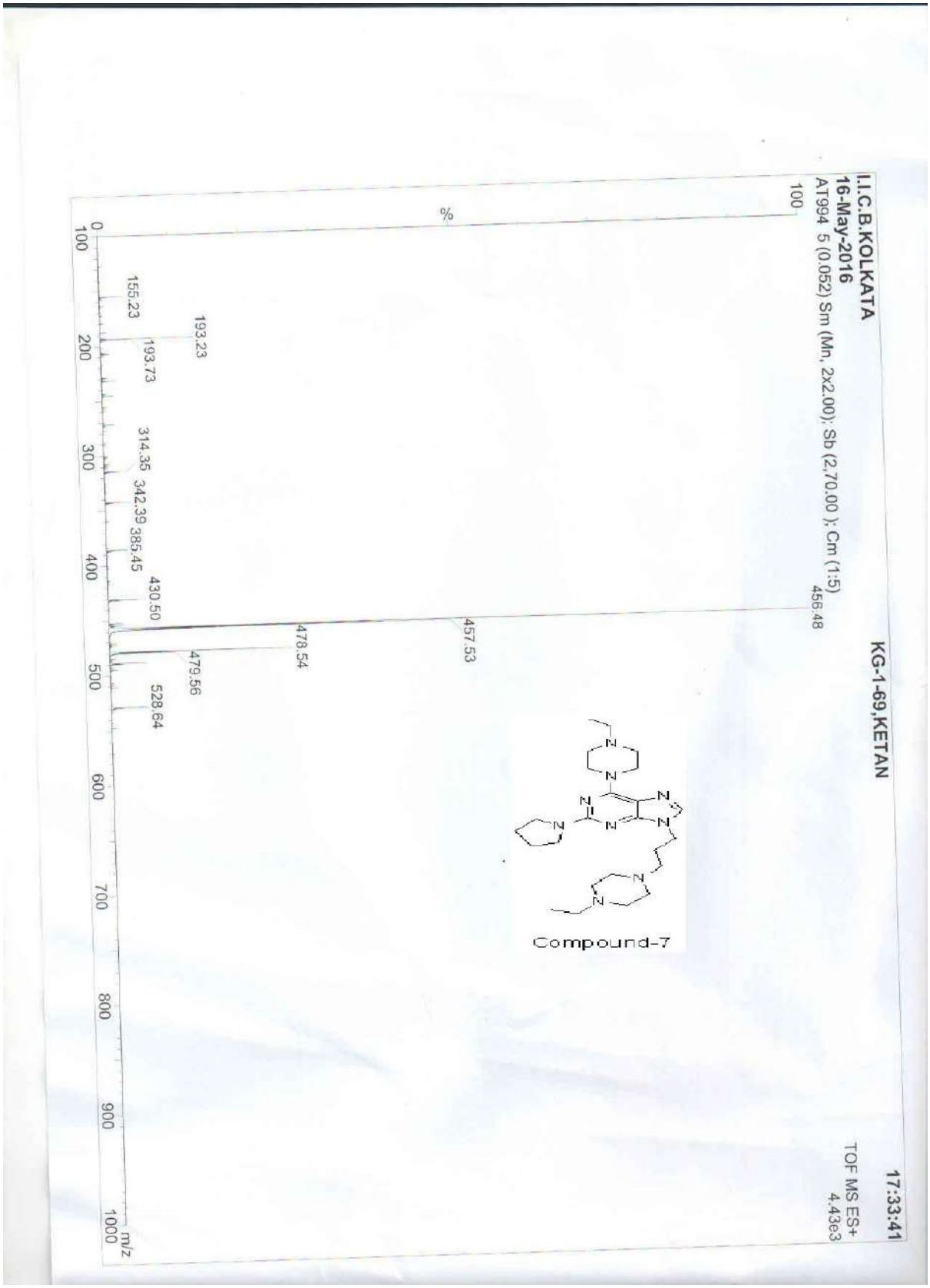

I.I.C.B.KOLKATA
14:40:46
AT305 8 (0.073) Sm (Mn, 1x2.00); Sb (2,70.00); Cm (1:8)
TOF MS ES+
1.09e3
100
%
0
248.20
177.11
177.47
178.12
205.15
248.60
270.19
270.59
271.21
517.35
100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 m/z
Compound-2

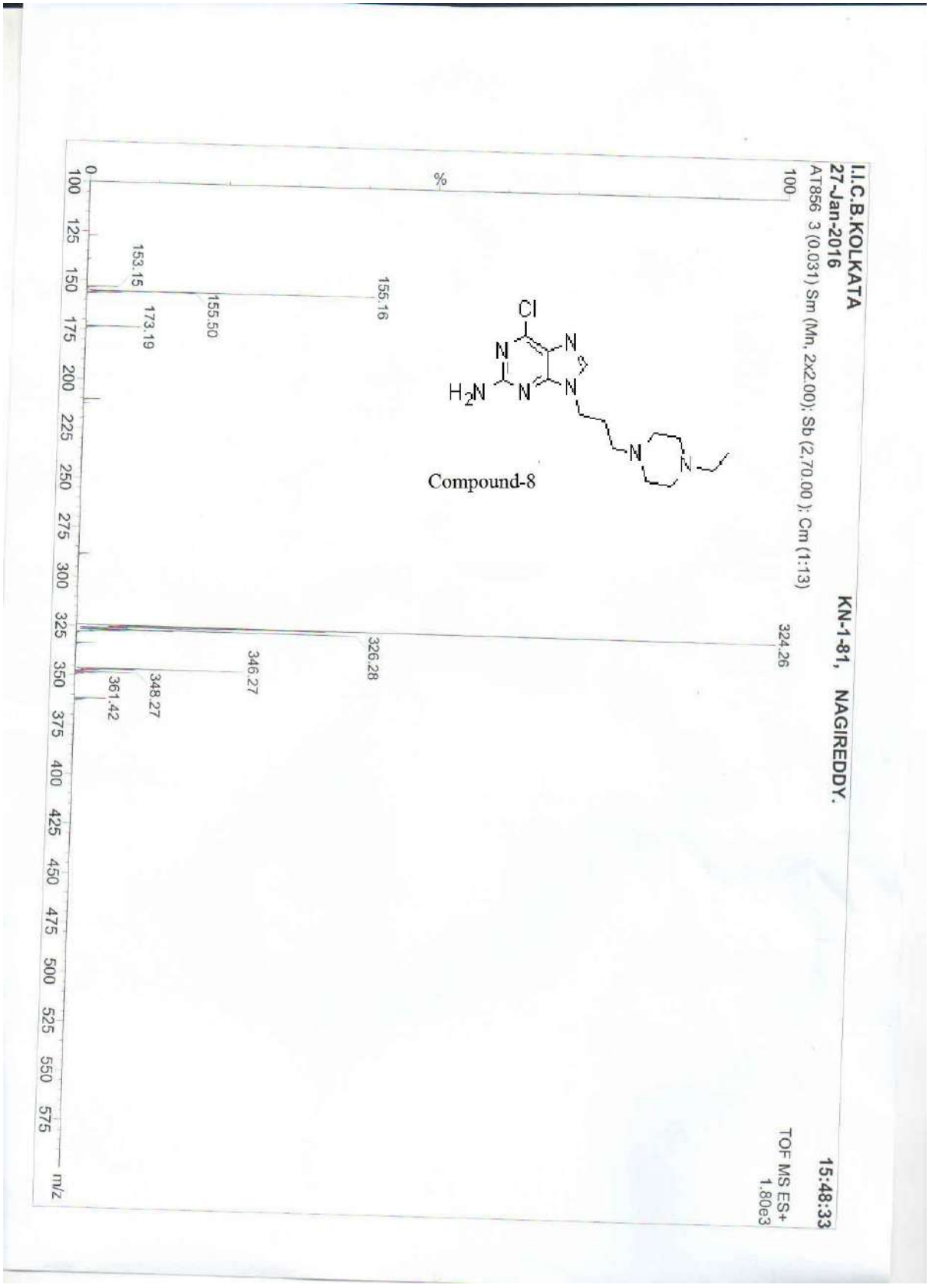

I.I.C.B.KOLKATA
16:08:23
AT440 5 (0.049) Sm (Mn, 2x1.00); Sb (2.70,00); Cm (1:6)
TOF MS ES+
4.26e3
%
100
0
324.43
253.35
255.34
288.85
289.47
288.45
326.88
327.49
326.46
Compound-3
Cl
m/z

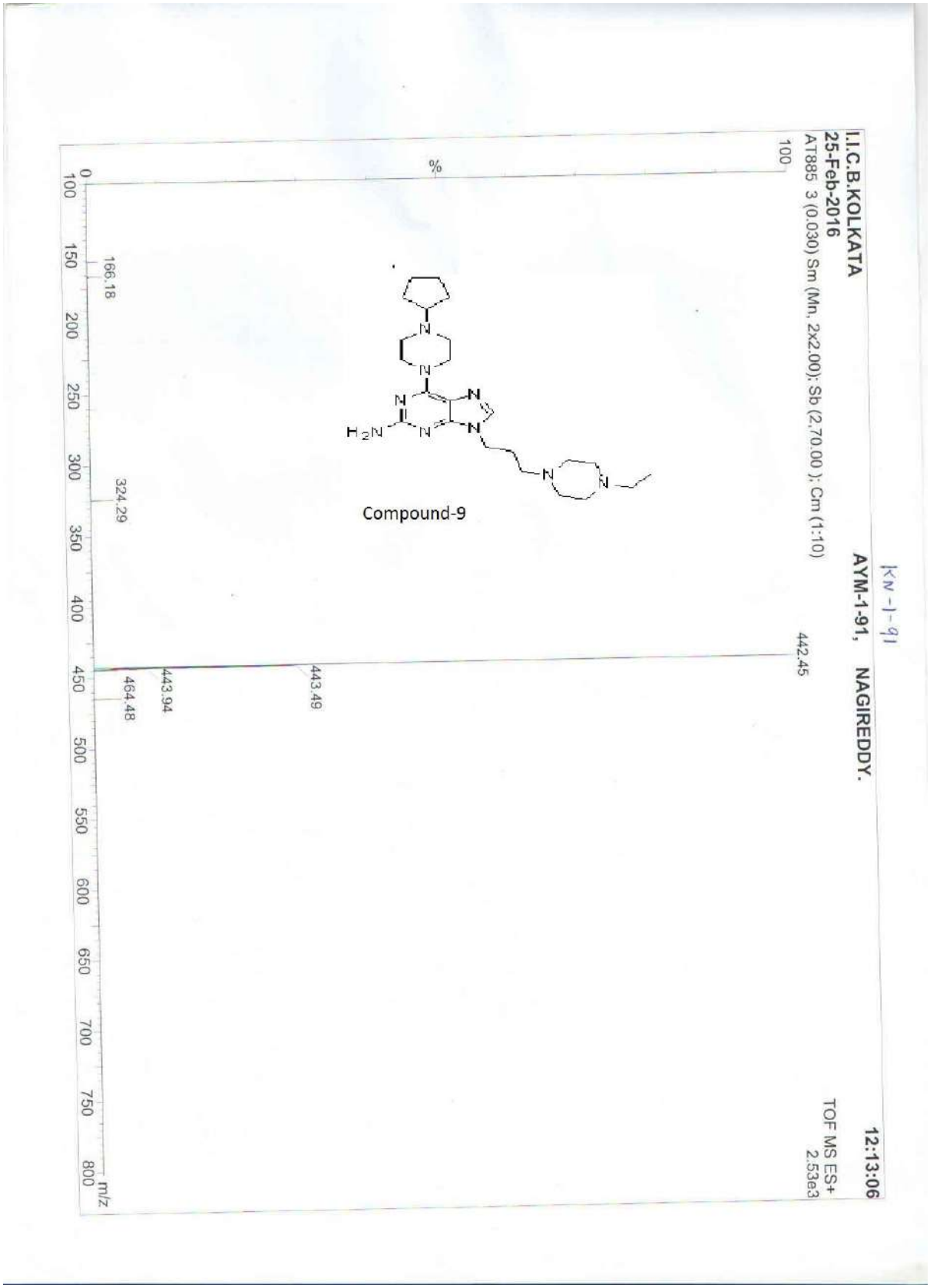

I.I.C.B.KOLKATA
AT321 2 (0.019) Sm (Mn, 1x2.00); Sb (2.70,00); Cm (2:6)
14:34:05
TOF MS ES+
1.71e3
%
m/z
288.37
402.47
424.52
425.53
Compound-4

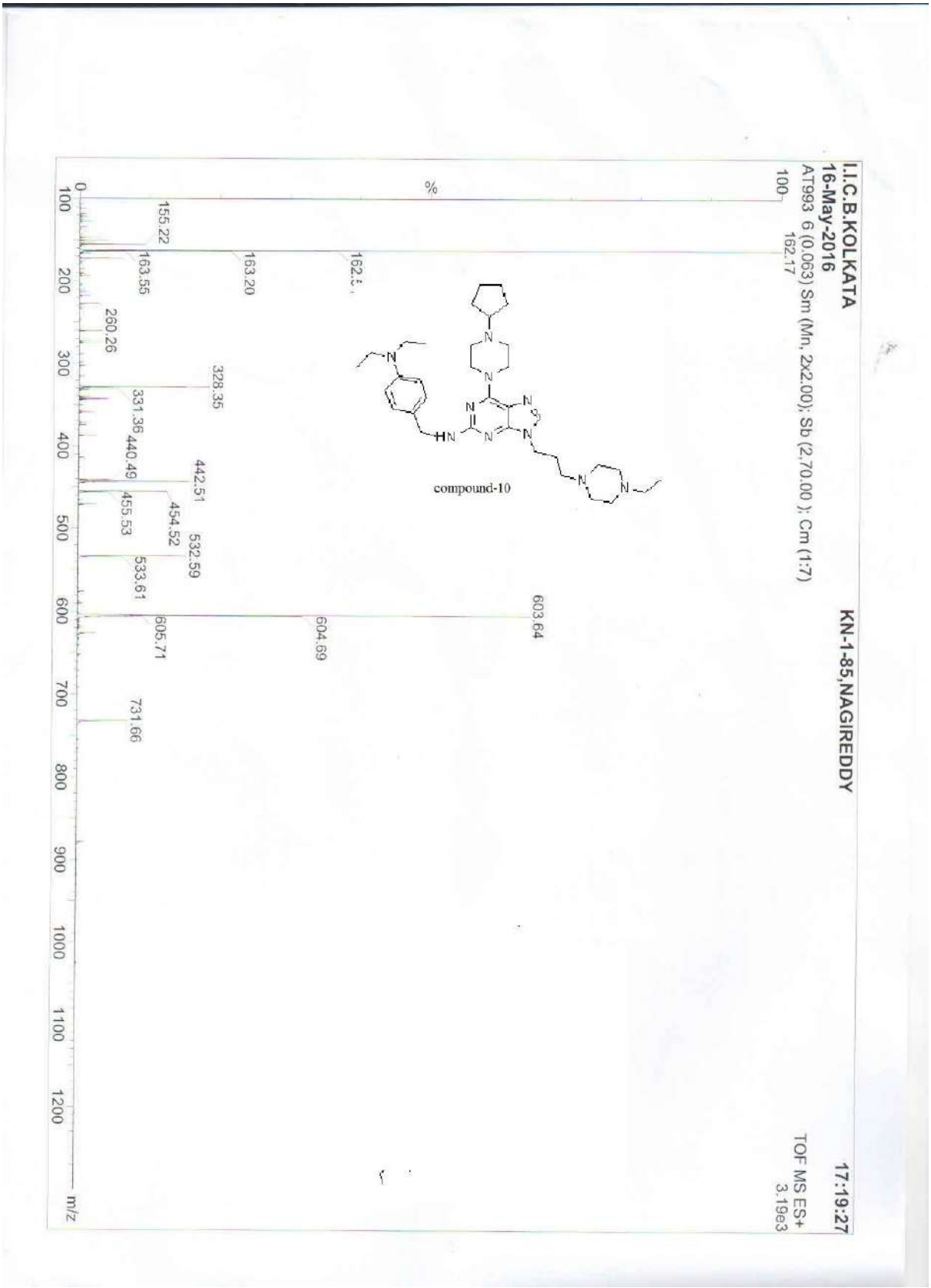

I.I.C.B.KOLKATA
16-Mar-2016
AT917 3 (0.031) Sm (Mn, 2x2.00); Sb (1,70.00); Cm (1:9)
KN-1-107, NAGIREDDY.
17:09:17
TOF MS ES+
1.42e3
%
m/z
261.81
331.41
402.51
522.60
523.63
524.09
544.64
MeO
NH
Compound-5

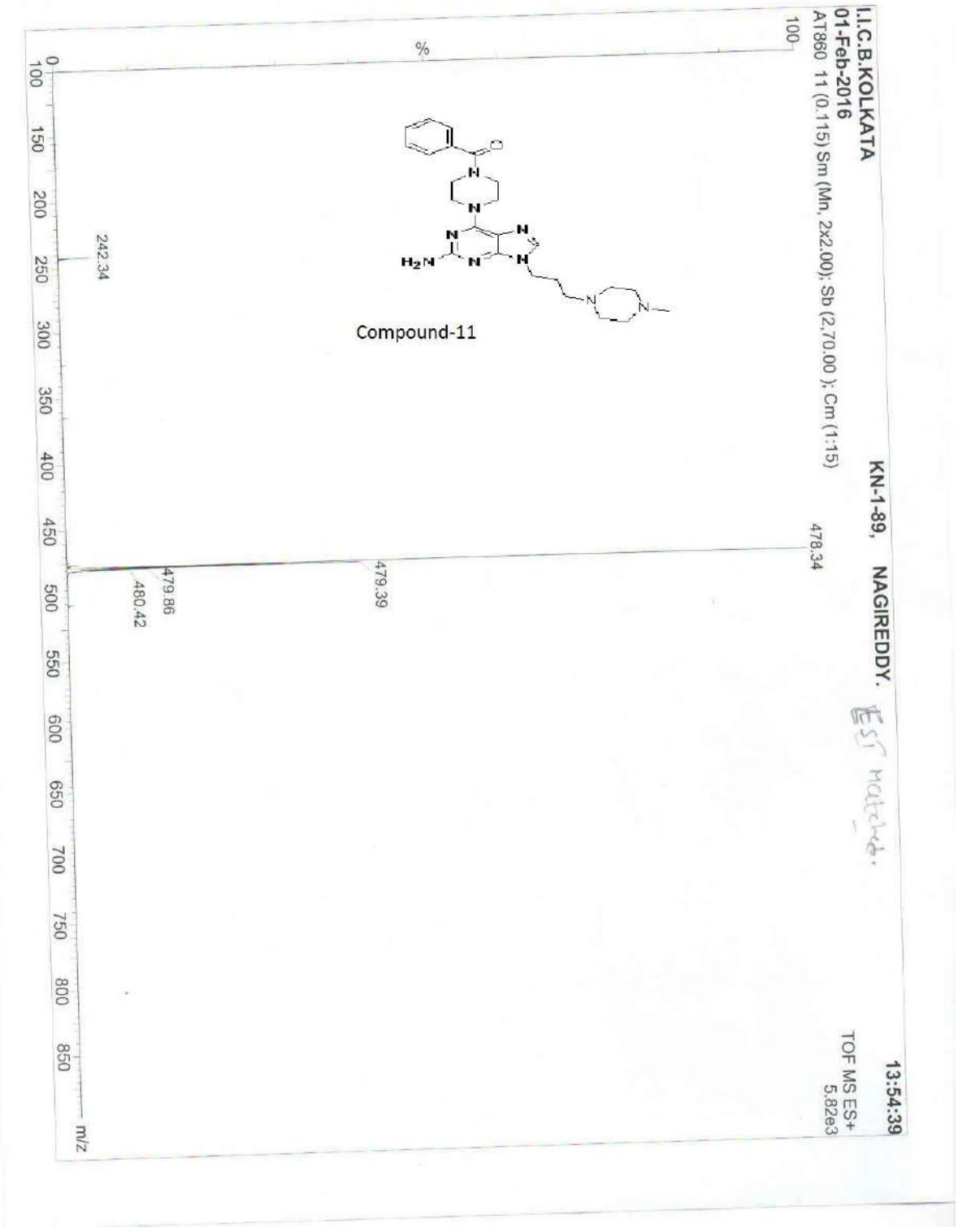

I.I.C.B.KOLKATA
22-Mar-2016
AT922 4 (0.042) Sm (Mn, 2x2.00); Sb (1,70.00); Cm (1:4)
KN-1-109,NAGIREDDY
10:59:23
TOF MS ES+
2.36e3
%
m/z
155.23
396.40
510.45
511.53
532.53
533.55
F
N
H
6
Compound-6

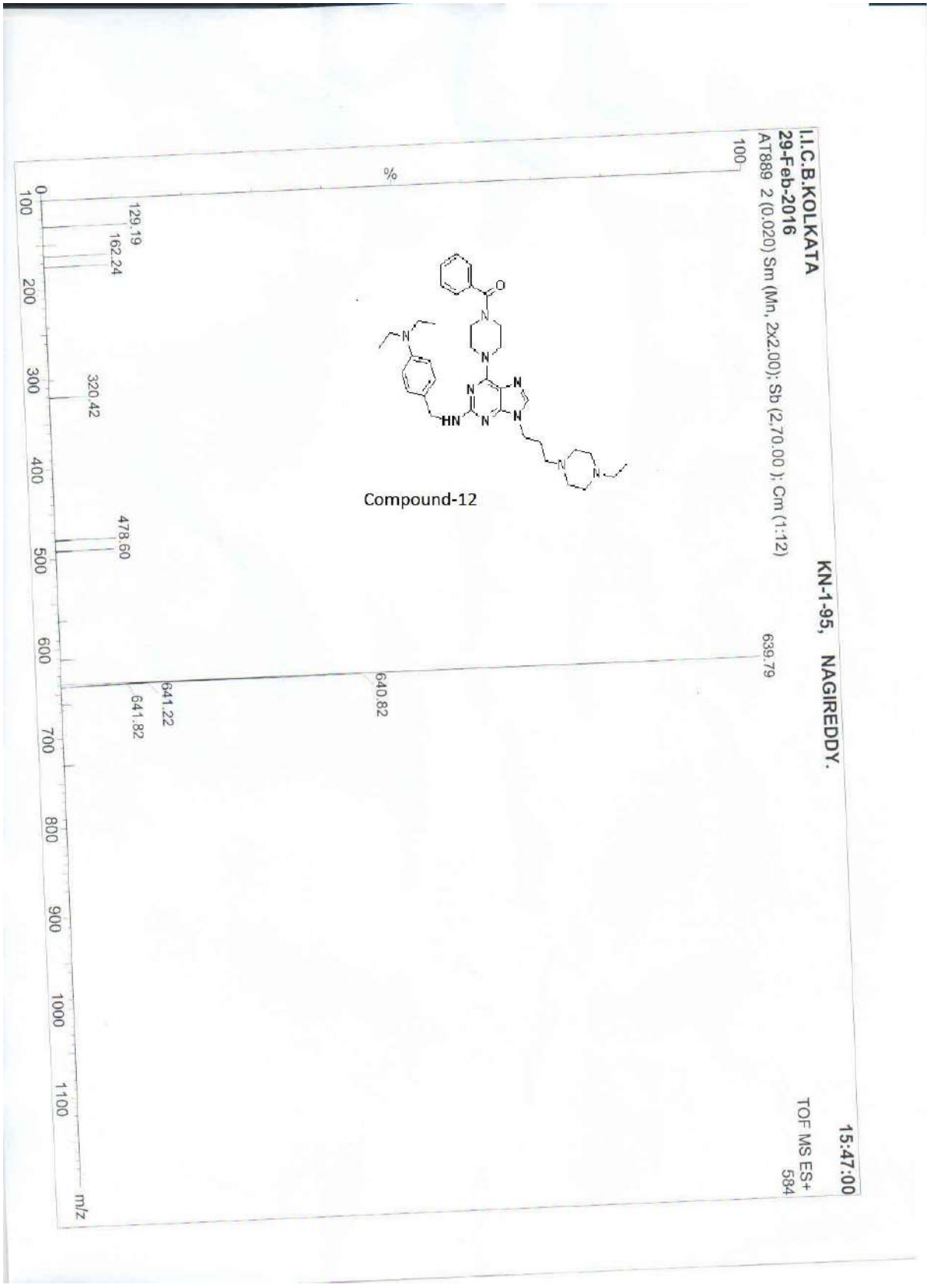

I.I.C.B.KOLKATA
16-May-2016
AT994 5 (0.052) Sm (Mn, 2x2.00); Sb (2,70.00); Cm (1:5)
KG-1-69,KETAN
17:33:41
TOF MS ES+
4.43e3
%
100
0
100
200
300
400
500
600
700
800
900
1000
m/z
155.23
193.23
193.73
314.35
342.39
385.45
430.50
456.48
457.53
478.54
479.56
528.64
Compound-7

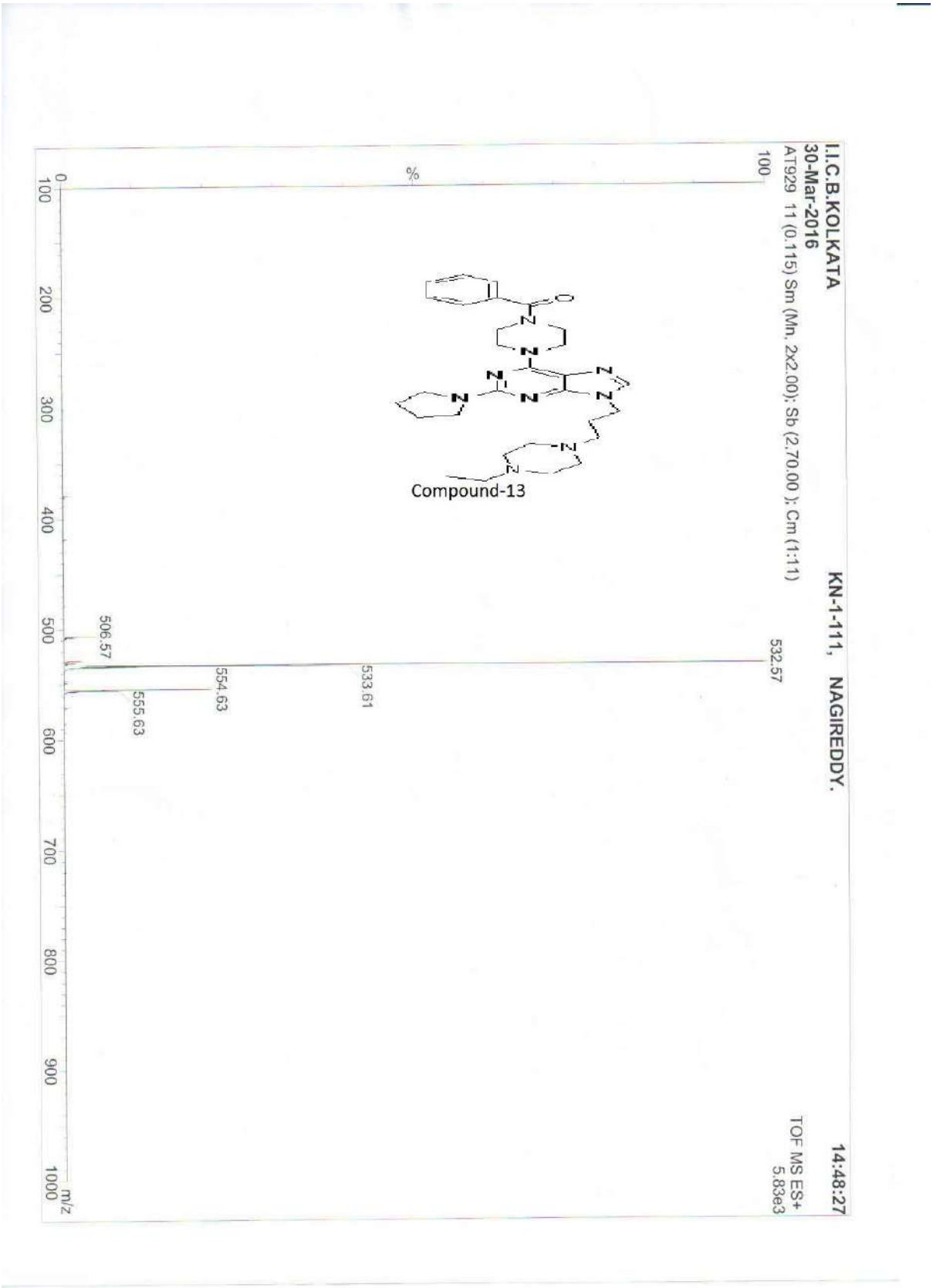

I.I.C.B.KOLKATA
27-Jan-2016
AT856 3 (0.031) Sm (Mn, 2x2.00); Sb (2.70,00); Cm (1:13)
KN-1-81, NAGIREDDY.
15:48:33
TOF MS ES+
1.80e3
%
100
0
155.16
153.15
173.19
155.50
324.26
326.28
346.27
348.27
361.42
100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575
m/z
Cl
N
N
N
S
N
H_2N
N
N
N
Compound-8


I.I.C.B.KOLKATA
25-Feb-2016
AT885 3 (0.030) Sm (Mn, 2x2.00); Sb (2,70.00); Cm (1:10)
AYM-1-91, NAGIREDDY.
12:13:06
TOF MS ES+
2.53e3
KN-1-91
%
100
0
166.18
324.29
442.45
443.49
443.94
464.48
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
m/z
Compound-9


I.I.C.B.KOLKATA
16-May-2016
AT993 6 (0.063) Sm (Mn, 2x2.00); Sb (2,70.00); Cm (1:7)
KN-1-85,NAGIREDDY
17:19:27
TOF MS ES+
3.19e3
%
m/z
compound-10
162.17
155.22
163.55
163.20
260.26
328.35
331.36
440.49
442.51
454.52
455.53
532.59
533.61
603.64
604.59
605.71
731.66


I.I.C.B.KOLKATA
01-Feb-2016
AT860 11 (0.115) Sm (Mn, 2x2.00); Sb (2,70.00); Cm (1:15)
KN-1-89, NAGIREDDY.
13:54:39
TOF MS ES+
5.82e3
ESI Matched.
%
100
0
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
m/z
242.34
478.34
479.39
479.86
480.42
Compound-11


I.I.C.B.KOLKATA
29-Feb-2016
AT889 2 (0.020) Sm (Mn, 2x2.00); Sb (2,70.00); Cm (1:12)
KN-1-95, NAGIREDDY.
15:47:00
TOF MS ES+
584
%
100
0
129.19
162.24
320.42
478.60
639.79
640.82
641.22
641.82
100
200
300
400
500
600
700
800
900
1000
1100
m/z
Compound-12


I.I.C.B.KOLKATA
30-Mar-2016
AT929 11 (0.115) Sm (Mn, 2x2.00); Sb (2.70,00); Cm (1:11)
KN-1-111, NAGIREDDY.
14:48:27
TOF MS ES+
5.83e3
Compound-13
%
m/z
506.57
532.57
533.61
554.63
555.63