Synthesis and Evaluation of Small Molecules Targeting miRNA-21

Thesis Submitted Towards Partial Fulfilment of

BS-MS Dual Degree program

By

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CERTIFICATE

This is to certify that this dissertation entitled **Synthesis and Evaluation of Small Molecules Targeting miRNA-21** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Tirukoti Deva Nishanth**, IISER Pune under the supervision of **Prof. Srinivas Hotha**, Associate Professor, Department of Chemistry, IISER Pune during the academic year 2015-2016.

Date: 25.04.2016
Place: Pune

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DECLARATION

I hereby declare that the matter embodied in the report entitled **Synthesis and Evaluation of Small Molecules Targeting miRNA-21** is the results of the chemistry carried out by me in the Department of Chemistry, IISER Pune, under the supervision of Prof. Srinivas Hotha and biological studies were carried out by me at CSIR-IGIB, New Delhi under supervision of Dr. Souvik Maiti. The same has not been submitted elsewhere for any other degree.

Date: 25.04.2016

Place: Pune

Tirukoti Deva Nishanth

Acknowledgments

I would like to thank my mentors Prof. Srinivas Hotha and Dr. Souvik Maiti for their support during the course of my masters' thesis, firstly I like to express my gratitude to Prof. Hotha who allowed me to carry project of my interest, with his immense support In academics and personally I am able to finish my project successfully. He pointed out my mistakes and corrected me towards betterment, I hope I will get same feedback and support from him in future.

I am highly thankful to my lab mates in IISER Pune for their support and I thank Dr.. Madhuri vangala for valuable suggestions during the course of my work, I like to mention specifically Dr. M. Ganesh Kumar from Dr. H.N. Gopi lab who also gave suggestions for successful completion of my project and Dr. E. Satish from Prof. K. N. Ganesh lab for the help in HPLC purification, I thank Bijoyananda Mishra for helping me to process NMRs and his support during my stay.

I like to extend my thanks to my lab mates in CSIR-IGIB, New Delhi because of the healthy environment in lab especially Smita Nahar. From performing the experiments together to have long discussions on miRNA, I thank for her patience for answering my basic questions in biology. I can't forgot the discussions I had with Soundhar Ramasamy who taught me how to use Confocal Microscopy and Zebra fish work.

I wholeheartedly, give my special thanks and sincere regards to all my friends and seniors in IISER Pune who taught me many things from personal to professional life and providing friendly environment during my 5 years of stay in IISER Pune.

All the above I would like to thank my parents and my sister for their emotional support during my BS-MS Degree in IISER Pune and in believing me what I do.

Tirukoti Deva Nishanth

Dedicated to Daddy and Amma

Contents

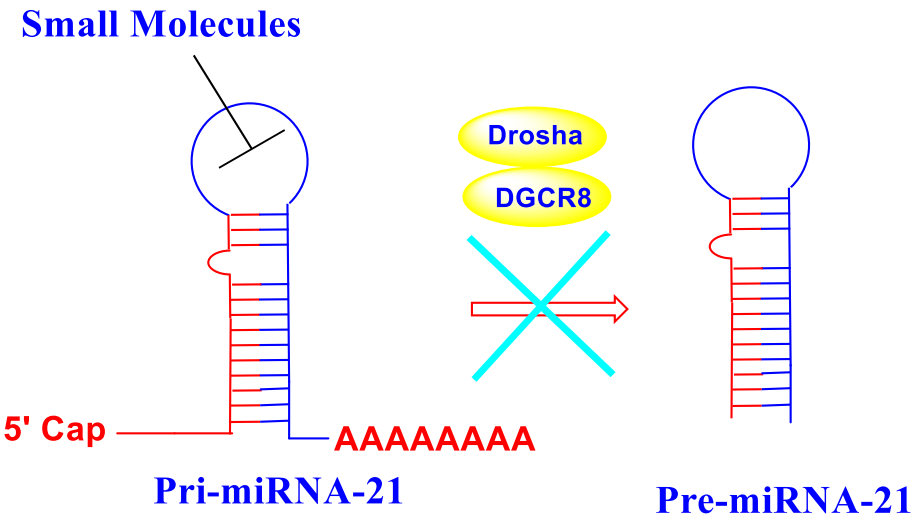
Abbreviations

TLC	:	Thin Layer Chromatography
NMR	:	Nuclear Magnetic Resonance
HRMS	:	High Resolution Mass Spectroscopy
MALDI	:	Matrix Assisted Laser Desorption/Ionization
J	:	Coupling Constant
Hz	:	Hertz
MHz	:	MegaHertz
DMF	:	*N,N'*-dimethylformamide
DCM	:	Dichloromethane
mg	:	Milligram
gm	:	Gram
h	:	Hour
M	:	Molar
mL	:	Milliliter
Mol	:	Mole
THF	:	Tetrahydrofuran
µM	:	Microliter
Boc	:	*Tert*-butoxycarbonyl
Fmoc	:	9-Fluorenylmethoxycarbonyl
Fmoc-OSu	:	N-(9-Fluorenylmethoxycarbonyloxy)succinimide
HBTU	:	*O*-Benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HPLC	:	High performance liquid chromatography
HOBt	:	Hydroxy Benzotriazole
TFA	:	Triflouro acetic acid
DIPEA	:	N,N-Diisopropylethylamine
PTSA	:	*p*-Toluenesulfonic acid
DIAD	:	Di-isopropyl azodicarboxylate
SDS	:	Sodiumdodecylsulfate
DMSO	:	Dimethyl sulfoxide
EDTA	:	Ethylenediaminetetraacetic acid

Abstract

miRNA-21 (miR-21) plays vital role in many cancers, inflammation, cardiovascular diseases. Targeting miR-21 is challenging due to the lack of bio-resistant oligo antisense nucleotides. To overcome the problem, developments of small organic molecules which inhibit the biogenesis pathway of miRNA are sought. In this study, we came with small molecule peptoids that target miRNA-21 biogenesis pathway and looked at their efficacy by carrying biological studies using the MCF7 cancer cell line.

Synthesis and Evaluation of Small Molecules Targeting miRNA-21



Introduction

MicroRNAs (miRNAs) are small class of non-coding RNA of ~20-22 nucleotides length which are conserved from viruses to animals. The main function of miRNAs is to target mRNA via complementary base pairing leading to translational arrest and repression of protein synthesis. They play crucial roles in most cellular pathways and development processes. They also have been identified to play pivotal roles in the cancer development and have been shown to act as oncogenes. They regulate processes such as proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism in cancer.[2] For example miRNA-21 acts as an oncogene by resisting apoptosis, [3] however there are miRNAs which can act as tumour suppressors. For example miRNA-29a can act as tumour suppressor leading to the tumour necrosis.[4] Modulation of miRNA levels in cancer cells by using therapeutic agents such as antisense oligonucleotides, modified oligonucleotides with phosphate backbone are excellent. Due to the poor stability of oligonucleotide and development of the resistance prompted development of small molecules as novel therapeutic agents.

miRNAs are transcribed by RNA polymerase II and to generate long primary transcripts (Pri-miRNA) that are typically polyadenylated at the end with one or more hairpin structures (Figure 1). Microprocessor complex assisted by Drosha and DGCR8 cleaves pri-miRNA in the nucleus to generate precursor miRNA (pre-miRNA) of ~70 nt.[5] The resulting double stranded pre-miRNA is transported to the cytoplasm by another protein called as the exportin-5 (Exp-5) (Figure 1). Exp-5 passes pre-miRNA to the Dicer complex which recognizes the 3'-end generated by the Drosha and cleaves the pre-miRNA into two helical turns in order to produce miRNA and miRNA*. Mature miRNA forms are incorporated into the RNA-induced silencing complex (RISC) and mediate the repression of translation by binding to the 3’-UTR region of target mRNA which is also referred to as Translational Repression.

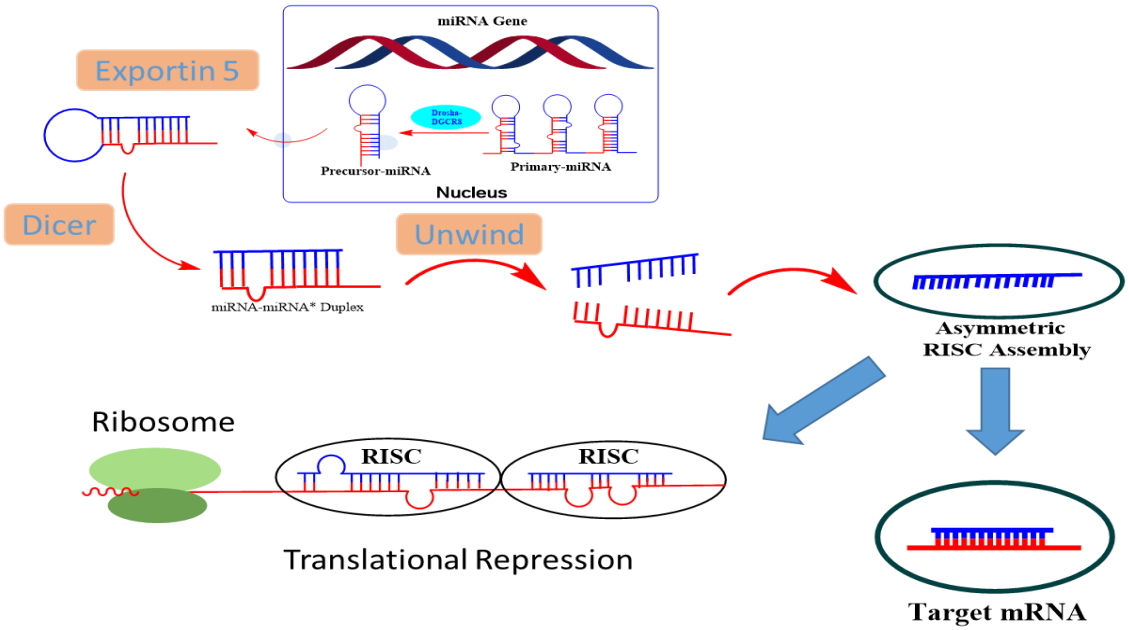


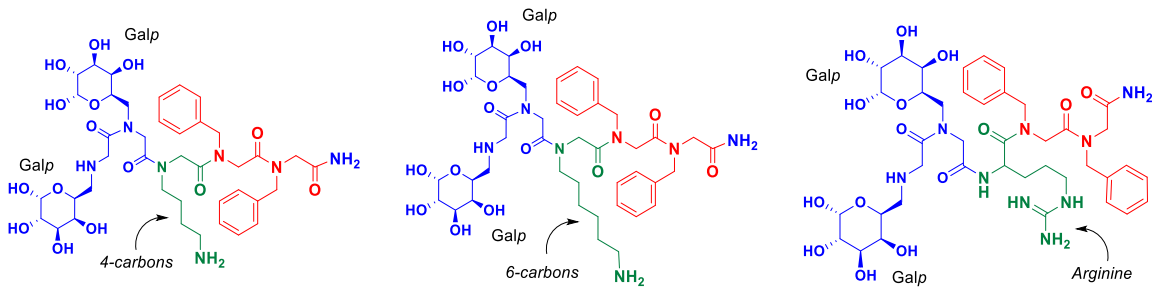
Figure 1: Biogenesis of miRNAs

Interestingly, wide range of miRNAs that map to the region of the human genome are frequently deleted or amplified in the cancer cells opening a new window of opportunity for targeting miRNAs by small molecules as **RNA Therapeutics**.

Recently, miRNA-21 has been revealed to play crucial roles in many biological processes and diseases including cell death, angiogenesis, activating invasion, metastasis, cardiovascular and inflammation.[6] In addition, oncogene miRNA-21 was shown to involve in the programed cell death 4 (Pdcd4)[7] and it is upregulated in many cancers and hence, small molecules that bind to miRNA-21 could become potential therapeutic agents in order to reduce their cellular concentrations. The specific aim of this project is to target Pri-miRNA- 21 with small molecules.

Design and Rationale of the Small Molecule Inhibitors for miRNA-21:

The microarray work by Sara Chirayil led to the discovery of small peptoids as potential binders to the apical loop of Pri-miRNA 21.[8] Continued interest in non-nucleotide small molecule binders for the miRNAs encouraged to look at the *in vivo* binding affinity of the identified peptoids to the miRNA-21. The microarray showed miRNA-21 binding efficiencies and they did not report any in vivo studies. It is well documented that miRNA-21 plays many roles including the tumor growth and thus, in vivo studies are highly rewarding for taking those peptoids further. To modulate the efficacy of the peptoids reported by Sara et al. we have increased the number of carbons in one of the monomers and the decreased the same number of carbons in the guanidine monomer in order to facilitate us the synthesis of desired peptoids using naturally occurring arginine.



P1 P2 P3

Solid Phase Peptide Synthesis (SPPS):

Emil Fisher and Vignead in 1901 made the synthesis of peptides possible for first time, [9] Fisher synthesised the first gly.gly dipeptide and fifty years later, du Vignead developed a strategy for the synthesis of polypeptides.[10] For synthesis of any polypeptide demands the use of orthogonal protecting groups on amino acids for the regio and chemoselective activation of the amine and the carboxylic acid present in an amino acid. Post-synthesis purification of large peptides in regular homogenous media is a herculean task and to facilitate easy purification, Bruce Merrifield in 1963 introduced synthesis of peptides on polymeric resins as an alternative. Synthesis of peptides on resins or solids became popular as solid phase peptide synthesis and several manufacturers currently sell machine that can synthesize peptides in an automated manner. This revolutionized and facilitated the development of peptide or protein science significantly.[12] The general work flow of solid phase synthesis is depicted in figure 2.

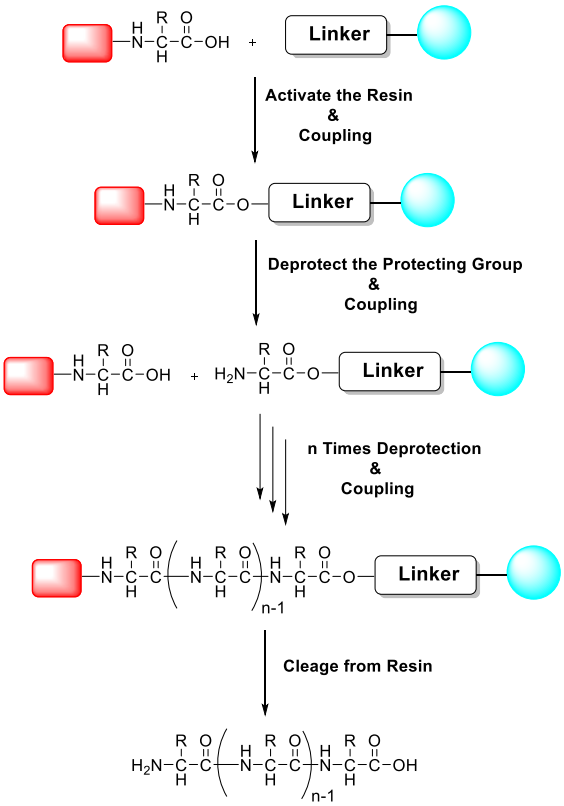
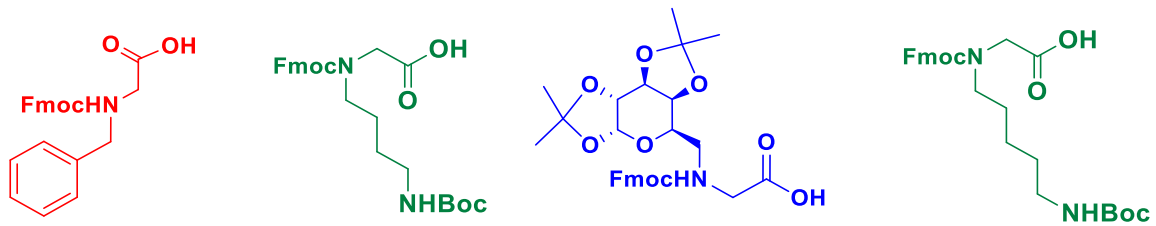


Figure 2: Flow sheet for the Merrifield's solid phase peptide synthesis

Commercially available resins are subjected to the deprotection of the end-functional group and then installed the first amino acid under standard peptide coupling conditions. The deprotection of the *N*-terminus, followed by the coupling of the next *N*-protected amino acid gives the dipeptide and the sequence is continued until the synthesis of identified peptide sequence is completed. Final cleavage of the desired peptide is performed in order to release the peptide from the polymeric resin. Several resins with diverse end-functional groups are commercially available. Resins are chosen depending on the stability of protecting groups present on the peptide molecules. There are resins that can be cleaved under strong to mild acidic and basic conditions and some resins can be cleaved off using neutral molecules as well. If one uses the Boc-chemistry, then acid resistant resin shall be used and the Fmoc-chemistry requires base resistant and acid labile resin.

The retrosynthetic synthesis analysis identified building blocks M1-M4 for the synthesis of peptide P1-P3 and the use of Fmoc-chemistry and Rink amide resin.



M1 **M2** **M3** **M4**

Figure 3: Building blocks required for the synthesis of peptoids P1-P3

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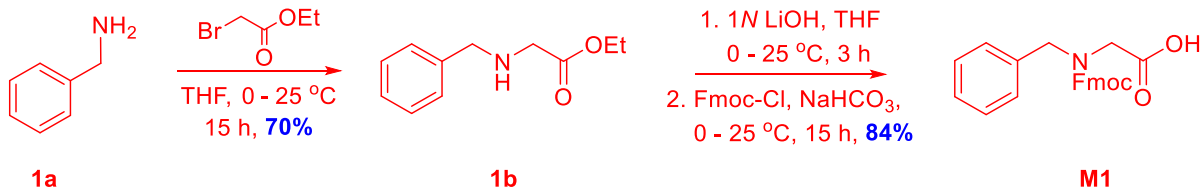
Results and Discussion

Targeted peptoids P1-P3 can be assembled by the use of four simple building blocks and the rink amide resin (Figure 3). The synthesis endeavour started with the preparation of monomers M1-M4 in large quantities before the commencement of solid phase synthesis. Fmoc-chemistry is invoked for the solid phase synthesis since the Boc-protecting group was present in M2 and M4 monomers.

Synthesis of **M1** was carried out in three simple steps,[13,14] *N*-Alkylation of benzyl amine was carried out with ethyl bromoacetate. The reaction was found to give mono- and di- N-alkylated products when the reaction was performed at higher temperatures; however, the mono-alkylated product 1b was predominant when the ethyl bromoacetate was added at 0 ^{o}C and the reaction mixture was slowly brought to the room temperature. Saponification of the ethyl ester with a 1N LiOH in THF for 3h afforded a carboxylic acid which was treated with Fmoc-Cl, $NaHCO_3$ in THF in order to obtain the required monomer M1 in 84% yield over two steps (Scheme 1).

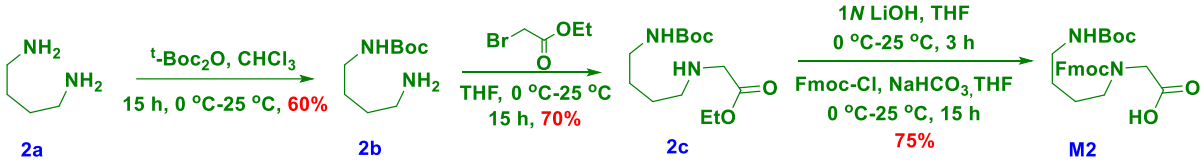
Scheme-1: Synthesis of M1



M1 were thoroughly confirmed by ^{1}H, ^{13}C NMR spectral analysis. For example, characteristic resonance at δ 174.9 ppm for C=O which is near to OH group, all the Fmoc group resonance in the region of (δ 120 ppm – 141.4 ppm). This confirms the formation of M1.

Synthesis of **M2** was achieved in four steps.[15,16,17] Commercially available 1, 4-diamino butane was treated with one equivalent of Boc_2O to obtain mono-carbamate (**2b**) in 60% yield. *N*-Alkylation under aforementioned conditions resulted in the ester **2c,** that was saponified and treated with Fmoc-Cl to afford the monomer M2 in good yield (Scheme 2).

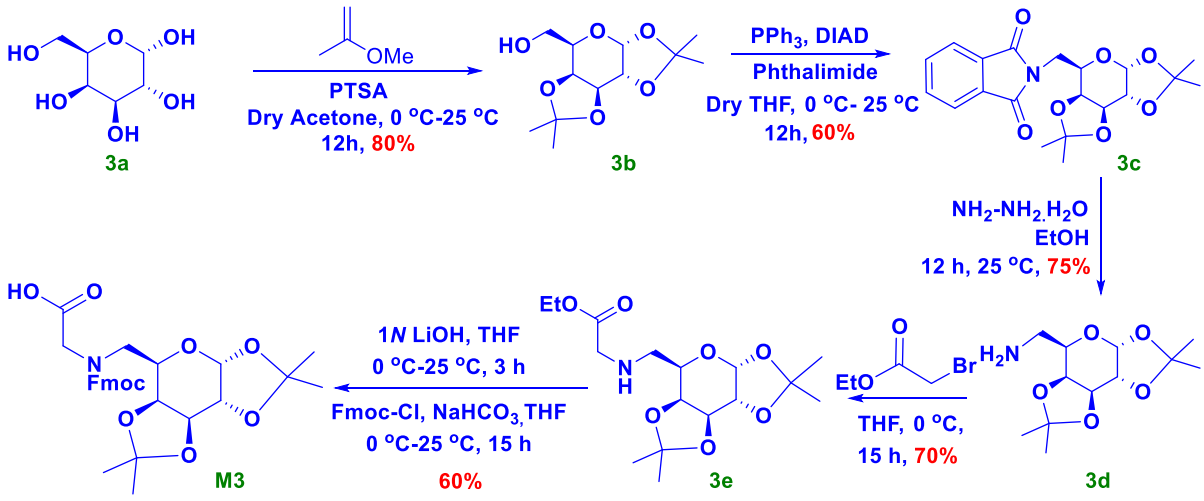
Scheme-2: Synthesis of M2



M2 were thoroughly confirmed by ^{1}H, ^{13}C NMR spectral analysis. For example, characteristic resonance at δ 173.5 ppm for C=O which is near to OH group and resonance at δ 79.3 for the quaternary carbon in the Boc, Fmoc resonance in the region of (δ 119.9 ppm – 143.9 ppm). This confirms the formation of M2**.**

Synthesis of **M3** was carried out in six steps.[18] D-galactose was converted into its 1, 2:3, 4-di-*O*-diacetonide using 2-methoxy propene and PTSA in anhydrous acetone. Conversation of the remaining C-6 hydroxyl group was treated with Ph_3P/DIAD/phthalimide to obtain the phthalimide derivative **3c**. The phthalimide **3c** was transformed into amine **3d** by the use of hydrazine hydrate followed by the *N*-alkylation using ethyl bromoacetate resulted in the isolation of compound **3e**. Saponification with LiOH followed by the Fmoc protection afforded the required monomer M3 in very good yield (Scheme-3).

Scheme-3: Synthesis of M3

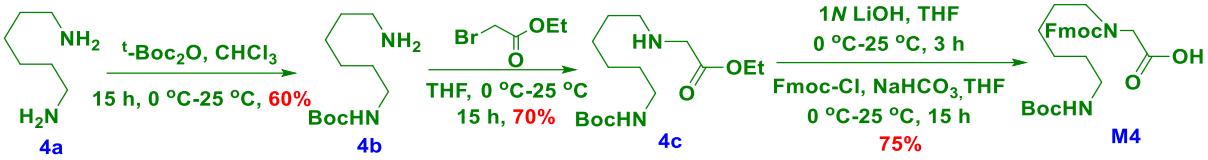


M3 were thoroughly confirmed by ^{1}H, ^{13}C NMR spectral analysis. For example, characteristic resonance at δ 99.27 ppm for anomeric carbon in the galactose and resonance at δ 174.7 ppm for C=O which is near to OH group and resonance at δ 79.3

for the quaternary carbon in the Boc, Fmoc resonance in the region of (δ 120 ppm – 144.3 ppm). This confirms the formation of M3**.**

Synthesis of **M4** was achieved in four steps.[19] Commercially available 1, 4-diamino butane was treated with one equivalent of Boc_2O to obtain mono-carbamate (**4b**) in 60% yield. *N*-Alkylation under aforementioned conditions resulted in the ester **4c** that was saponified and treated with Fmoc-Cl to afford the monomer M4 in good yield (Scheme 4).

Scheme-4: Synthesis of M4

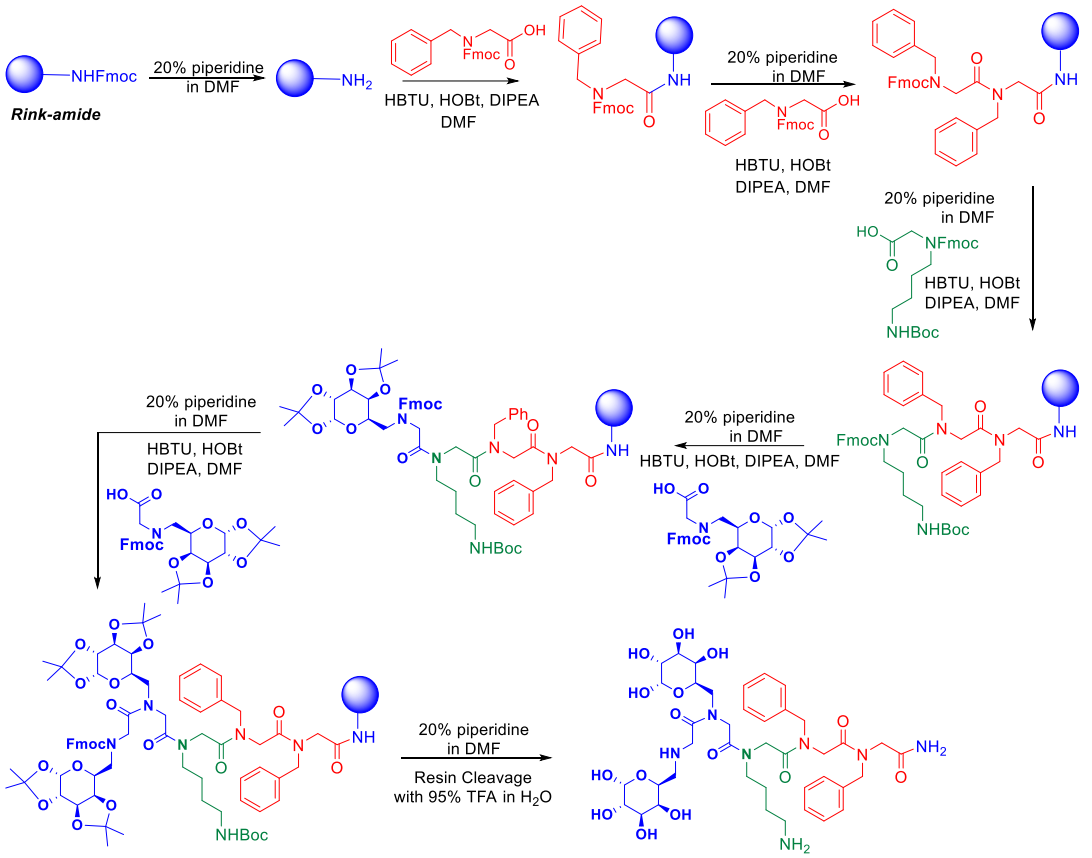


M4 were thoroughly confirmed by ^{1}H, ^{13}C NMR spectral analysis. For example, characteristic resonance at δ 173.7 ppm for C=O which is near to OH group and resonance at δ 79.3 for the quaternary carbon in the Boc, Fmoc resonance in the region of (δ 119.9 ppm – 144.0 ppm). This confirms the formation of M4**.**

Solid phase synthesis of **P1**:

Successful synthesis of monomers M1, M2, M3 set the stage for the solid phase peptide synthesis. Rink amide resin in DMF was treated with 20% (v/v) piperidine in DMF to get the resin bound free amine. Free amine resin was treated with monomer-1, HBTU, HOBt, DIPEA in DMF to get the first peptoid on to the resin. The progress of the reaction was monitored by the well documented Kaiser test. Subsequently, deprotection of Fmoc moiety was achieved by the treatment of 20% (v/v) piperidine in DMF and the next monomer M2 was attached with standard reaction conditions using HBTU, HOBt, and DIPEA in DMF. Fmoc deprotection followed by the amide bond formation with M3 monomer gave the resin containing tri-peptoid. Repetition of these steps resulted in the successful synthesis of desired peptoids (Scheme 5). Formation of the peptoid-1 was confirmed by MALDI-TOF.

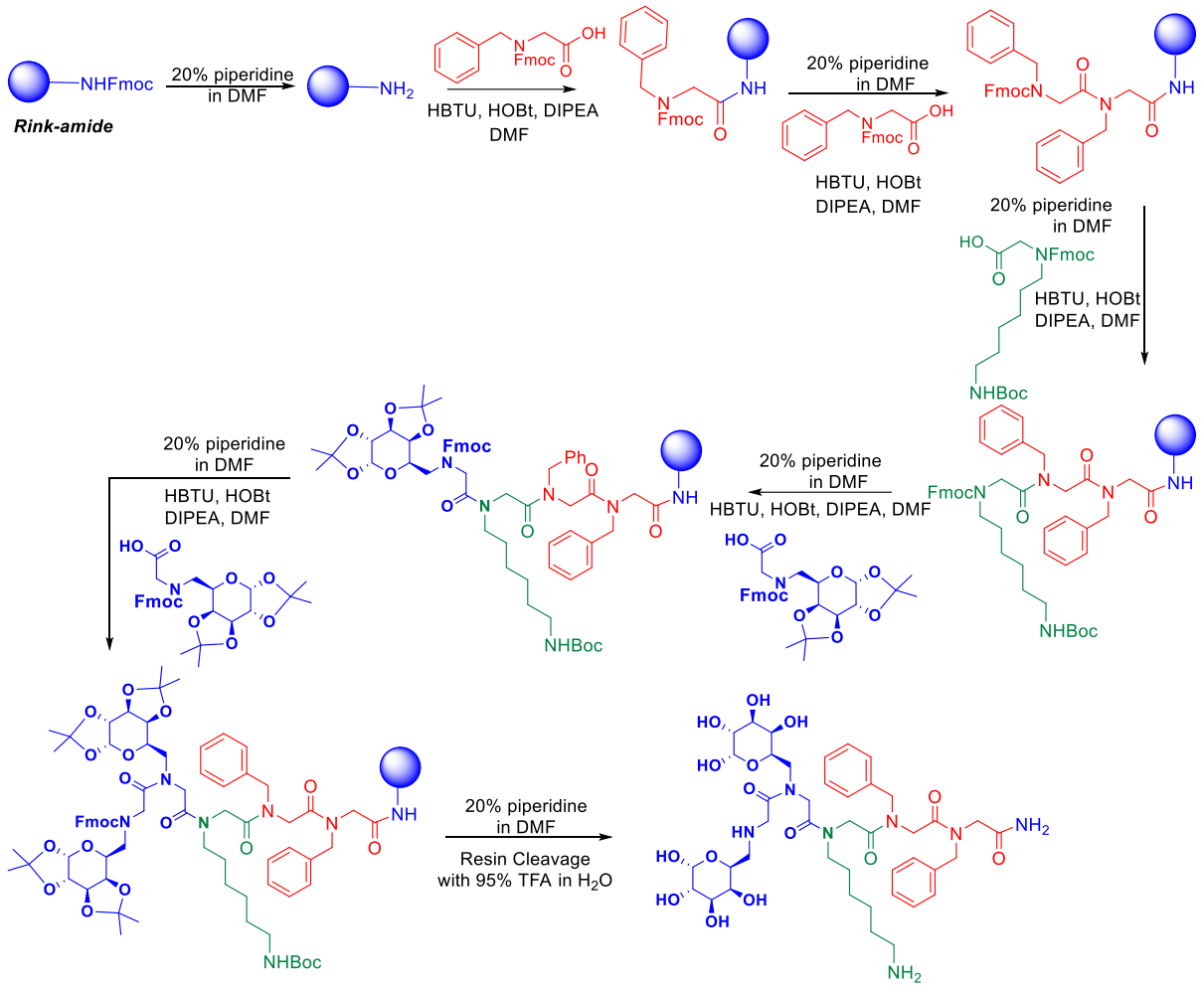
Scheme 5: Synthesis of P1 by SPPS



Solid phase synthesis of **P2**:

Successful synthesis of monomers M1, M4, M3 set the stage for the solid phase peptide synthesis. Rink amide resin in DMF was treated with 20% (v/v) piperidine in DMF to get the resin bound free amine. Free amine resin was treated with monomer-1, HBTU, HOBt, DIPEA in DMF to get the first peptoid on to the resin. The progress of the reaction was monitored by the well documented Kaiser test. Subsequently, deprotection of Fmoc moiety was achieved by the treatment of 20% (v/v) piperidine in DMF and the next monomer M4 was attached with standard reaction conditions using HBTU, HOBt, and DIPEA in DMF. Fmoc deprotection followed by the amide bond formation with M3 monomer gave the resin containing tri-peptoid. Repetition of these steps resulted in the successful synthesis of desired peptoids (Scheme 6). Formation of the peptoid-2 was confirmed by MALDI-TOF.

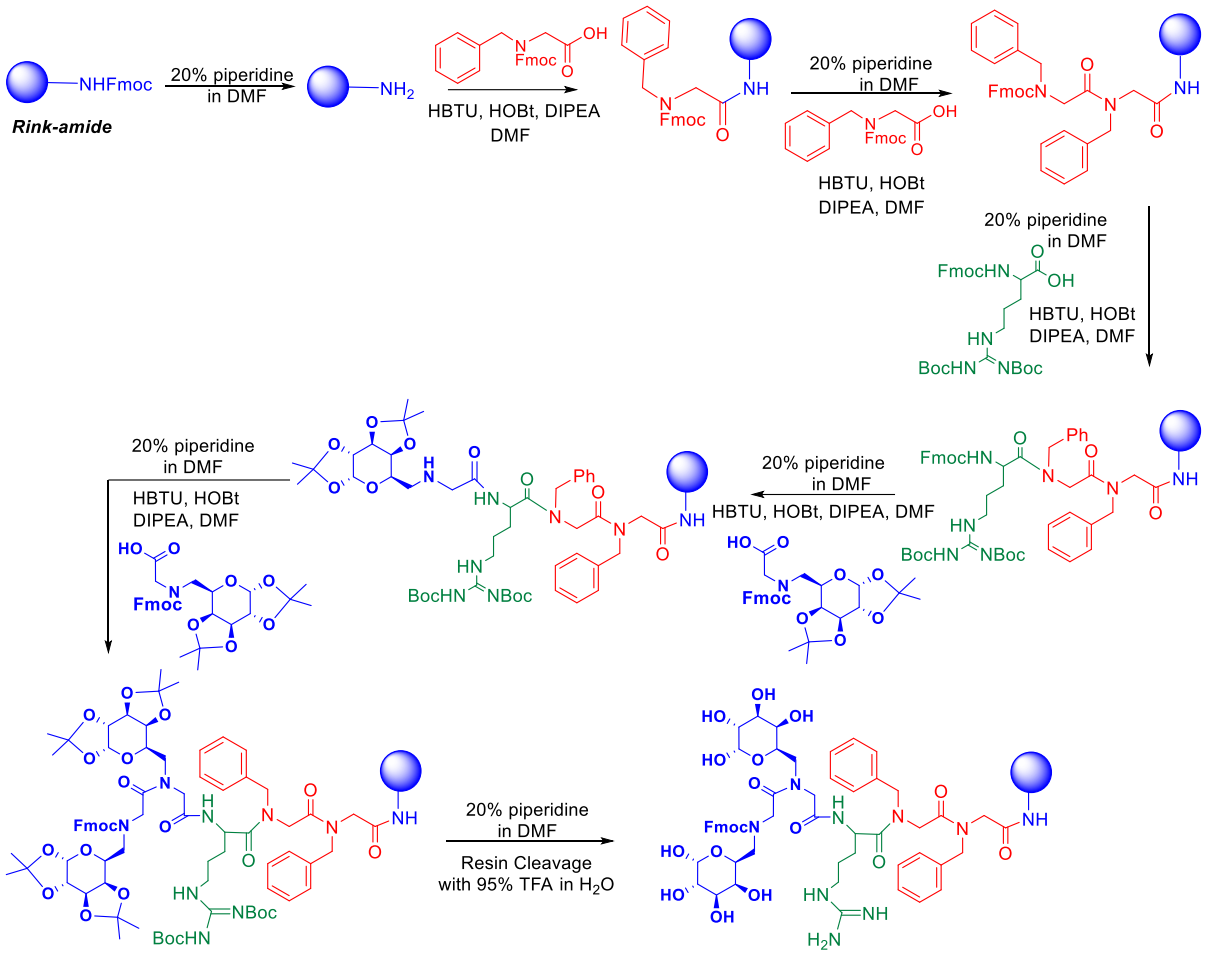
Scheme 6: Synthesis of P2 by SPPS



Solid phase synthesis of **P3**:

Successful synthesis of monomers M1, M3 set the stage for the solid phase peptide synthesis. Rink amide resin in DMF was treated with 20% (v/v) piperidine in DMF to get the resin bound free amine. Free amine resin was treated with monomer-1, HBTU, HOBt, DIPEA in DMF to get the first peptoid on to the resin. The progress of the reaction was monitored by the well documented Kaiser test. Subsequently, deprotection of Fmoc moiety was achieved by the treatment of 20% (v/v) piperidine in DMF and the Boc protected arginine was attached with standard reaction conditions using HBTU, HOBt, and DIPEA in DMF. Fmoc deprotection followed by the amide bond formation with M3 monomer gave the resin containing tri-peptoid. Repetition of these steps resulted in the successful synthesis of desired peptoids (Scheme 7). Formation of the peptoid-3 was confirmed by MALDI-TOF.

Scheme 7: Synthesis of P3 by SPPS



Peptoids P1-P3 was cleaved from the Rink amide resin using 95% TFA in Water. Crude peptoids were purified by using preparative HPLC with gradient binary mobile phase system. Chromatograms were recorded at λ 254nm for the aromatic rings and 220 nm for the peptide bond.

Solvent system A: (95% Acetonitrile: 5% H_2O: 1% TFA)

Solvent system B: (95% H_2O: 5% Acetonitrile: 1% TFA)

Gradient Method:

Time	Solvent System A	Solvent System B
0-5 min	0 %	100 %
5-20 min	50%	50%
20-25min	0%	100%

Eluted compounds were concentrated *in vacuo* and subjected to lyophilisation to obtain pure peptoids P1-P3 as white powders.

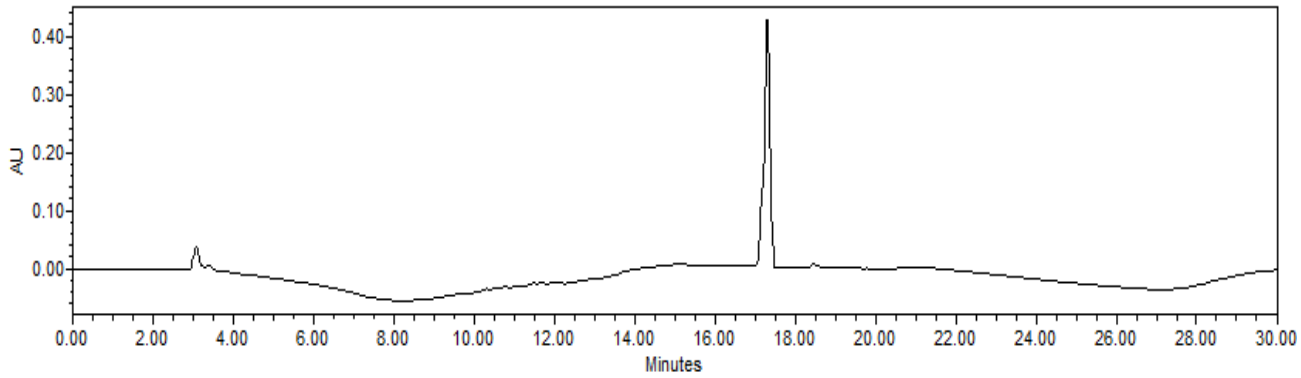


Figure 4: HPLC chromatogram of **P1**

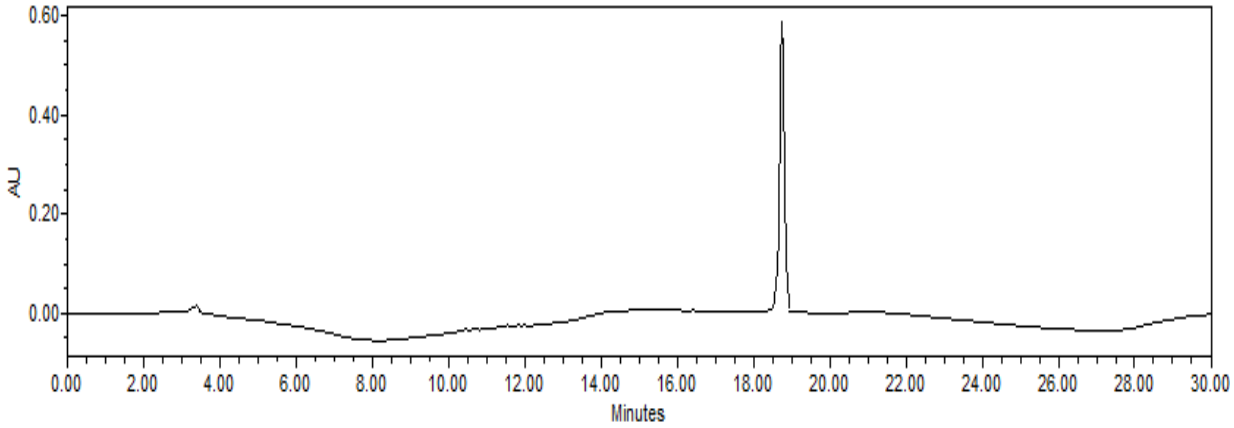


Figure 5: HPLC chromatogram of **P2**

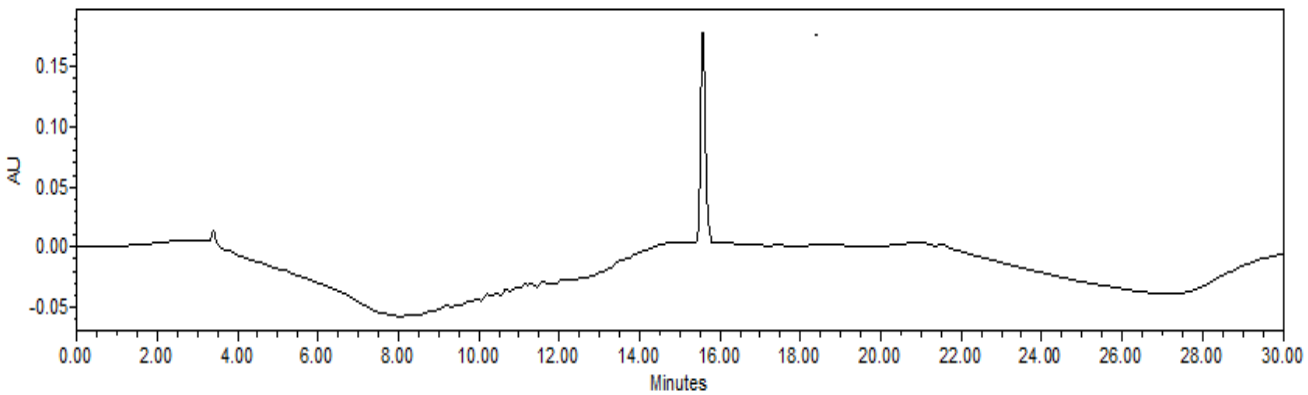


Figure 6: HPLC chromatogram of **P3**

		Expected	Observed
P1	$(M+H^+)$	878.41	878.52
	(M+Na)	900.39	900.51
	(M+K)	916.37	916.48
P2	$(M+H^+)$	906.4460	906.5842
	(M+Na)	928.4280	928.5401
	(M+K)	944.4019	944.4642
P3	$(M+H^+)$	906.42	906.44
	(M+Na)	928.40	928.38

Figure 7: P1, P2, P3 Confirmation by MALDI-TOF

Successful synthesis of **P1**-**P3** peptoids encouraged us to check their biological significance. To measure the cytotoxicity, MTT assay was performed on **P1**-**P3** wherein the luminescence by formazan crystals was measured to obtain the IC_{50} value. NADPH dependent oxidoreductase enzyme which is present in Mitochondria of the living cells has capability to reduce the tetrazolium dye to purple colour formazan crystal in the living cells. If the cells die then there will be no production of the oxidoreductase enzyme and thus no formation of formazan crystals, and hence no change in the colour intensity. MTT assay was performed on all the three peptoids at different concentrations *viz*. 1 µM, 2 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 200 µM, 500 µM, 1000 µM in a 96 well plate. All the experiments were performed in triplicate and average values were taken. MTT data clearly suggested that the peptoids P1-P3 have minimal influence on cell viability.

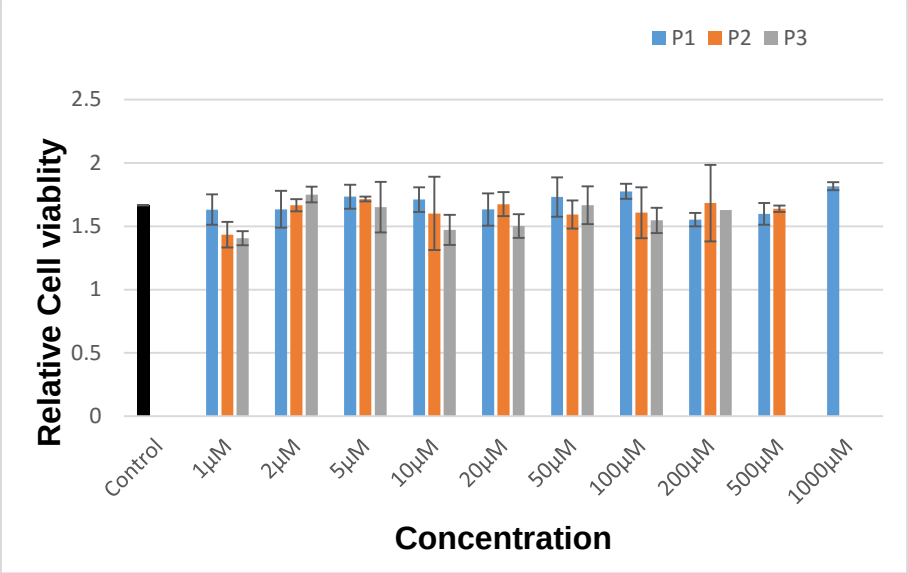


Figure 8: Effect of Concentration on relative cell viability in MTT assay conditions

In continuation, influence of the peptoids P1-P3 on miRNA-21 levels by gene reporter assay (Dual Luciferase assay) was investigated. Plasmids were designed in such a way that codons for both luciferase and miRNA-21 at the 3'-UTR end are present. If peptoids reduced the maturation level of the miRNA-21, then there shall be down regulation of miRNA-21 and as a consequence anticodons for the plasmid will diminish. Hence, upregulation of proteins will be seen and hence intensity of luciferase will increase. We treated MCF-7 cells with peptoids for 48 h at different concentrations (0.5 μM, 1 μM, 2 μM, 5 μM and 10 μM). Peptoid **P3** is anticipated to have more binding affinity due to the presence of guanidine group in the molecule and luciferase assay was carried out at high concentrations (20 μM, 50 μM, 100 μM) of **P3.**

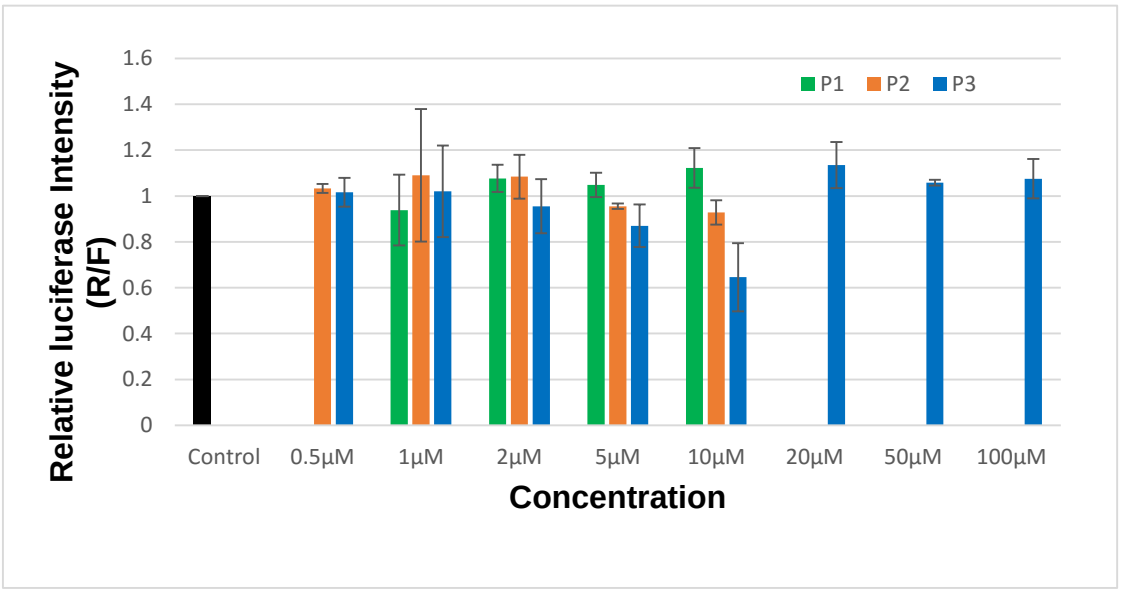


Figure-9: Luciferase Levels after 48 hrs Treatment

Cells which are incubated with only plasmid are called as vector control that acts as a reference to check the luminescence level with the effect of peptoids. It is welcoming to see that peptoids P1, P2, P3 showed response to the luminescence assay conditions. Peptoid **P1-P3** showed negligible improvement in the luciferase intensities suggesting tested peptoids are not really suitable for further investigation even at 10 µM. In desperation, we have tried higher concentration for **P3** to observe no additional improvement.

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Materials and Methods

- All the chemicals for peptoid synthesis are purchased from Sigma-Aldrich, Rankem or Spectrochem.

- All the chemicals for buffers and culture media constituents to grow MCF-7 cells were purchased from Sigma-Aldrich and Thermo Fisher.

- ^{1}H NMR, ^{13}C NMR and DEPT spectra were recorded on Jeol-400 MHZ or Bruker 400 MHz spectrometers using tetramethylsilane (TMS) as an internal standard.

- MALDI data were recorded on Applied Biosystems MALDI-TOF Mass Spectrometer.

- All reactions were monitored by Thin Layer Chromatography (TLC) carried out on 0.25 mm E-Merck silica gel plates (60G F-254) with UV-light, staining with I_2 and anisaldhyde in ethanol or ninhydrine or Phosphomolybdic acid stain (PMA).

- Unless otherwise mentioned, all the reactions were conducted under inert Argon atmosphere with anhydrous solvents purchased from Merck and Finar.

- All the evaporations were carried out under reducing pressure on Heidolph rotary evaporator below 40 ^{0}C unless otherwise specified.

- HPLC Purification was performed on Reverse Phase C_{18} column.

- Silica gel 100-200 mesh was used for column chromatography.

Experimental Procedure

Ethyl benzylglycinate **(1b):** A round bottom flask containing benzyl amine (**1a**) (2.0 gm, 18.6 mmol) in anhydrous THF (30 mL) was stirred at 0^0 C for 5 min and ethyl bromoacetate (1.8 mL, 16.8 mmol) in 7 mL anhydrous THF was added to the reaction mixture in dropwise fashion. After 15 h, the reaction mixture was concentrated *in vacuo* and the resulting crude residue was purified by silica gel on column chromatography using ethyl acetate and petroleum ether as mobile phase to obtain compound **1b** (2.52 gm, 70%) as yellow oil.

N-(((9H-fluoren-9-yl)methoxy)carbonyl)-*N*-benzylglycine (**M1**): To a solution of compound **1b** (2.0 gm, 10.35 mmol) in THF (20 mL) at 0 ^{0}C was added 5 mL of aqueous 1M LiOH dropwise and stirred at 25 ^{o}C for 3h. After completion of the reaction (adjudged by 30% EtOAc in light petroleum ether), reaction mixture was concentrated under reduced pressure, acidified with Amberlite resin at pH 6 and carried forward for the next step without further purification. To the crude residue (1.71 gm, 10.35 mmol) in THF (20 mL), 5 mL of 10% Na_2CO_3 (aqueous) was added at 0 ^{o}C and was stirred for 10 min. Fmoc-Cl (2.68 gm, 10.35 mmol) in THF (5 mL) and added to the reaction mixture by dropwise. The reaction mixture was stirred for 15h and diluted with water, extracted with ethyl acetate (25 mL) to remove excess Fmoc-Cl. The aqueous layer was acidified with dil HCl, extracted with ethyl acetate (3 x 25 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated under reduced pressure and the crude residue was purified by silica gel (100-200 mesh) column chromatography using ethyl acetate and petroleum ether to obtain monomer **M1** (3.35 gm, 85%) as a white solid (mp = 120-127 ^{o}C); ^{1}H NMR (400.31 MHz, $CDCl_3$) δ 3.75 (s, 1H), 3.99 (s, 1H), 4.23 (dt, *J* = 12.1, 6.2 Hz, 1H), 4.48 (s, 1H), 4.55 (d, *J* = 6.1 Hz, 3H), 7.03 – 7.10 (m, 1H), 7.17 (d, *J* = 6.6 Hz, 1H), 7.21 – 7.32 (m, 6H), 7.31 – 7.40 (m, 2H), 7.49 (d, *J* = 7.5 Hz, 1H), 7.54 (d, *J* = 7.4 Hz, 1H), 7.72 (d, *J* = 7.5 Hz, 2H) ; ^{13}C NMR (100.67 MHz, $CDCl_3$) δ 46.8, 46.9 (CH), 47.1, 47.2, (CH_2), 51.2, 51.4 (CH_2), 67.7, 67.8 (CH_2), 68.1, 68.1 (CH_2),120, 120 (CH),120.0, 120.0 (CH), 124.8, 124.9 (CH), 124.9, 124.9 (CH), 127.1, 127.1 (CH), 127.1, 127.1 (CH), 127.6, 127.6 (CH), 127.7, 127.7 (CH), 127.8, 127.8 (CH), 127.8, 127.8 (CH), 127.8, 127.8 (CH), 128.2, 128.2 (CH), 128.8, 128.8 (CH), 128.8, 128.8 (CH), 136.4, 136.4 (CH), 141.4,

143.7 (C), 143.8,143.8 (C) 156.4, 156.7(C=0), 174.9, 175.0(C=O); HRMS (Waters Synapt G2): m/z calcd for $[C_{24}H_{21}NO_4 + H]^+$: 388.1549; Found: 388.1545.

tert-Butyl (4-aminobutyl) carbamate (**2b**)**:** A round bottomed flask containing compound (**2a**) (1.0 gm, 11.34 mmol) in $CHCl_3$ (10 mL) was stirred at 0 ^{O}C, di-tert-butyl dicarbonate (2.0 mL, 9.08 mmol) in $CHCl_3$ (5 mL) was added to the reaction mixture. The reaction mixture was stirred in room temperature for 15 h and concentrated *in vacuo* to obtain a residue that was silica gel (100-200 mesh) column chromatography using methanol and chloroform as mobile phase to obtain compound **2b** (1.2 gm, 60%) as a thick syrup.

Ethyl (4-((tert-butoxycarbonyl)amino)butyl)glycinate (**2c**): A round bottomed flask containing compound (2b) (1.0 gm, 5.31 mmol) in anhydrous THF (15 mL) was stirred at 0^0 C for 5 min, ethyl bromo acetate (0.5mL, 4.78 mmol) in 5 mL anhydrous THF was added to the reaction mixture in dropwise fashion. After 15 h reaction mixture was concentrated *in vacuo* and the resulting crude mixture was purified by silica gel on column chromatography using methanol and chloroform as mobile phase obtained compound **2c** (1.2 gm, 70%) as yellow syrup.

N-(((9H-fluoren-9-yl) methoxy) carbonyl)-N-(4-((tert-butoxycarbonyl) amino) butyl) glycine (**M2**): To a solution of compound **2c** (1.0 gm, 3.64 mmol) in THF (20 mL) at 0 ^{0}C was added 5 mL of aqueous 1M LiOH dropwise and stirred at 25 ^{o}C for 3h. After completion of the reaction (adjudged by 30% MeOH in chloroform), reaction mixture was concentrated under reduced pressure, acidified with Amberlite resin at pH 6 and carried forward for the next step without further purification. To the crude residue (0.879 gm, 3.64 mmol) in THF (20 mL), 5 mL of 10% Na_2CO_3 (aqueous) was added at 0 ^{o}C and was stirred for 10 min. Fmoc-Cl (0.942 gm, 3.64 mmol) in THF (5 mL) and added to the reaction mixture by dropwise. The reaction mixture was stirred for 15h and diluted with water, extracted with ethyl acetate (25 mL) to remove excess Fmoc-Cl. The aqueous layer was acidified with dil HCl, extracted with ethyl acetate (3 x 25 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated under reduced pressure and the crude residue was purified by silica gel (100-200 mesh) column chromatography using methanol and chloroform to obtain monomer **M2** (1.2 gm, 75%) as a white solid (mp = 117-123 ^{o}C); ^{1}H NMR (400.31 MHz, $CDCl_3$) δ 1.19 – 1.30 (m, 2H), 1.34 – 1.56 (m, 10H), 2.06 (d, *J* = 14.1 Hz, 1H), 2.92 – 3.15 (m,

3H), 3.32 (d, *J* = 7.1 Hz, 1H), 3.92 (d, *J* = 18.5Hz, 2H), 4.11 (s, 1H), 4.18 – 4.23 (m, 1H), 4.41 (d, *J* = 6.0 Hz, 1H), 4.55 (d, *J* = 5.3 Hz, 1H), 7.23 – 7.43 (m, 4H), 7.54 (t, *J* = 6.8 Hz, 2H), 7.74 (dd, *J* = 12.0, 7.5 Hz, 2H); ^{13}C NMR (100.67 MHz, $CDCl_3$) 20.8, 21.1 (CH_2), 25.0, 25.3 (CH_2), 28.4, 28.4 (CH_3),28.4, 28.4 (CH_3), 28.4, 28.4 (CH_3), 47.3, 47.3 (CH_2),48.1, 48.1 (CH), 48.4, 48.4 (CH_2), 48.9, 49.0 (CH_2), 67.2, 67.7 (CH_2), 79.4, 79.4 (C), 119.9, 119.9 (CH), 119.9, 119.9 (CH), 124.7, 124.7 (CH), 125.0, 125.0 (CH), 127.1, 127.1 (CH), 127.1, 127.1 (CH), 127.6, 127.6 (CH), 127.7, 127.7 (CH), 141.3, 141.3 (C), 141.4, 141.4 (C), 143.9, 143.9 (C), 143.9, 143.9 (C), 156.1, 156.1 (C=O), 156.6, 156.6 (C=O), 173.5, 173.5 (C=O); HRMS (Waters Synapt G2): m/z calcd for $[C_{26}H_{32}N_2O_6 + H]^+$: 469.2339; Found: 469.2342.

1, 2:3, 4-di-O-isopropylidene galactopyranose (**3b**): To a suspension of galactose (5.0 gm, 27.75 mmol) in dry acetone (100 mL) at 0^0 C was added 2-methoxypropene (6.81 mL, 69.38 mmol) and stirred 10 min. Then, catalytic amount of PTSA was added to the reaction mixture and after 12 h, the reaction mixture was neutralized by the addition of triethyl amine. The reaction mixture was concentrated *in vacuo* to obtain a residue that was purified by silica gel column chromatography using ethyl acetate and petroleum ether as mobile phase to obtain compound **3b** (5.7 gm, 80%) as yellow syrup.

1,2:3,4-di-O-isopropylidene 6-*deoxy*-6-phthalimido galactopyranose (**3c**): A round bottom flask containing compound (**3b**) (5.0 gm, 19.21 mmol) in dry THF (40 mL) was stirred at 0 ^{o}C, triphenylphosphine (5.29 gm, 20.17 mmol), phthalimide (2.97 gm, 20.17 mmol) were added and stirred for 5 min. Diisopropyl azodicarboxylate (3.77 mL, 19.21 mmol) was added to the reaction mixture and stirred at 25 ^{o}C for 12 h. The yellow coloured reaction mixture was concentrated *in vacuo* and the crude residue was purified by silica gel (100-200 mesh) on column chromatography using ethyl acetate and petroleum ether to afford phthalimide derivative **3c** (4.4 gm, 60%) as a thick yellow syrup.

1, 2:3, 4-di-O-isopropylidene 6-*deoxy*-6-amino galactopyranose (**3d**): To a solution of compound (**3c**) (4.4 gm, 19.21 mmol) in EtOH (60 mL) was added hydrazine hydrate (2.48 mL) at 25 ^{o}C and vigorously stirred for 12 h. The resulting white precipitate of phthalizine was filtered through a pad of celite. The crude reaction mixture was taken directly for the the next step without further purification (2.0 gm, 75%).

1,2:3,4-di-O-isopropylidene 6-*deoxy*-6-ethyl glycinato galactopyranose (**3f**): A round bottom flask containing compound (**3b**) (2.0 gm, 7.71 mmol) in anhydrous THF (25 mL) was stirred at 0 ^{o}C for 5 min, ethyl bromo acetate (0.7mL, 6.94 mmol) in 7 mL anhydrous THF was added to the reaction mixture in dropwise fashion. After 15 h, reaction mixture was concentrated *in vacuo* and the resulting crude mixture was purified by silica gel column chromatography using ethyl acetate and petroleum ether as mobile phase to obtain compound **3f** (1.86 gm, 70%) as yellow syrup.

1,2:3,4-di-O-isopropylidene 6-*deoxy*-6-*N*-glycino galactopyranose (**M3**): To a solution of compound **3f** (1.8 gm, 5.21 mmol) in THF (10 mL) at 0 ^{o}C was added 2 mL of aqueous 1M LiOH dropwise and stirred at 25 ^{o}C for 3h. After completion of the reaction (adjudged by TLC analysis with 5% MeOH: 95% $CHCl_3$ mobile phase), reaction mixture was concentrated under reduced pressure, acidified with Amberlite resin at pH 6 and carried forward for the next step without further purification. To the crude residue (0.879 gm, 3.64 mmol) in THF (20 mL), 5 mL of 10% Na_2CO_3 (aqueous) was added at 0 ^{o}C and was stirred for 10 min. Fmoc-Cl (1.65 gm, 5.20 mmol) in THF (15 mL) was added to the reaction mixture dropwise. The reaction mixture was stirred for 15 h and diluted with water, extracted with ethyl acetate (25 mL) to remove excess Fmoc-Cl. The aqueous layer was acidified with dil HCl, extracted with ethyl acetate (3 x 25 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated under reduced pressure and the crude residue was purified by silica gel (100-200 mesh) column chromatography using ethyl acetate and petroleum ether as mobile phase to obtain monomer **M3** (1.6 gm, 60%) as a white fluffy compound (mp = 88-94 ^{o}C); ^{1}H NMR (400.31 MHz, $CDCl_3$) δ 1.11 – 1.53 (m, 12H), 3.30 (ddd,*J* = 23.2, 14.9, 8.5 Hz, 1H), 3.45 – 3.93 (m, 2H), 4.05 – 4.30 (m, 5H), 4.30 – 4.64 (m, 3H), 5.47 (dd, *J* = 10.3, 4.9 Hz, 1H), 7.23 – 7.42 (m, 4H), 7.50 – 7.61 (m, 2H), 7.73 (dd, *J* = 14.9, 7.5 Hz, 2H) ; ^{13}C NMR (100.67 MHz, $CDCl_3$) 24.3, 24.3 (CH_3), 25.0, 25.1 (CH_3), 26.0, 26.0 (CH_3), 26.1, 26.1 (CH_3), 47.2, 47.2 (CH), 49.1, 50.0(CH_2), 50.3, 50.7 (CH_2), 66.9, 67.4 (CH_2), 67.8, 68.0 (CH), 70.5, 70.6 (CH), 70.8, 70.8 (CH), 71.3, 71.5 (CH), 96.2, 96.3 (CH), 108.7, 109.0 (C), 109.4, 109.5 (C), 120.0, 120.0 (CH), 120.0, 120.0 (CH), 124.9,124.9 (CH), 125.0, 125.0 (CH), 125.0, 125.1 (CH), 125.1, 127.2 (CH), 127.3, 127.4 (CH), 127.8, 127.8 (CH), 141.3, 141.3 (C), 141.4, 141.4 (C), 143.8, 143.8 (C), 143.9, 143.9 (C), 156.1, 156.6 (C=O), 174.7, 174.8 (C=O); HRMS (Waters Synapt G2): m/z calcd for $[C_{29}H_{33}NO_9 + H]^+$: 540.2234; Found: 540.2231.

tert-butyl (6-aminohexyl) carbamate (**4b**): A round bottom flask containing compound (**4a**) (3.0 gm, 25.82 mmol) in $CHCl_3$ (30 mL) was stirred at 0 ^oC, di-tert-butyl dicarbonate (5.0 mL, 23.23 mmol) in $CHCl_3$ (5 mL) was added to the reaction mixture. The reaction mixture was stirred in room temperature for 15 h and concentrated *in vacuo* to obtain a residue that was subjected to the silica gel (100-200 mesh) column chromatography using methanol and chloroform as mobile phase to obtain compound **4b** (3.3 gm, 60%) as a yellow syrup.

Ethyl (6-((tert-butoxycarbonyl)amino)hexyl)glycinate (**4c**)**:** To a rapidly stirred solution of compound (**4b**) (3.0 gm, 13.87 mmol) in anhydrous THF (30 mL) at 0 ^oC, ethyl bromo acetate (1.53mL, 4.78 mmol) in 10 mL anhydrous THF was added in dropwise fashion. After 15 h, reaction mixture was concentrated *in vacuo* and the resulting crude mixture was purified by silica gel on column chromatography using methanol and chloroform to obtain compound **4c** (2.9 gm, 70%) as yellow solid.

(6-((tert-butoxycarbonyl)amino)hexyl)glycine (**M4**): To a solution of compound **4c** (2.5 gm, 8.27 mmol) in THF (15 mL) at 0 ^0C was added 3 mL of aqueous 1M LiOH dropwise and stirred at 25 ^oC for 3h. After completion of the reaction (adjudged by TLC), reaction mixture was concentrated under reduced pressure, acidified with Amberlite resin at pH 6 and carried forward for the next step without further purification. To the crude residue (2.2 gm, 8.02 mmol) in THF (15 mL), 5 mL of 10% Na_2CO_3 (aqueous) was added at 0 ^oC and was stirred for 10 min. Fmoc-Cl (2.0 g, 8.02 mmol) in THF (5 mL) and added to the reaction mixture by dropwise. The reaction mixture was stirred for 15 h and diluted with water, extracted with ethyl acetate (25 mL) to remove excess Fmoc-Cl. The aqueous layer was acidified with dil. HCl, extracted with ethyl acetate (3 x 25 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated under reduced pressure and the crude residue was purified by silica gel (100-200 mesh) column chromatography using ethyl acetate and petroleum ether to obtain monomer **M4** (2.5 gm, 70%) as a thick syrup. ^1H NMR (400.31 MHz, $CDCl_3$) δ 1.27 (d, *J* = 11.3 Hz, 6H), 1.44 (s, 14H), 3.10 (dt, *J* = 12.4, 6.6 Hz, 3H), 3.32 (s, 1H), 3.94 (d, *J* = 11.0 Hz, 2H), 4.15 – 4.26 (m, 1H), 4.38 (d, *J* = 6.6 Hz, 1H), 4.49 (d, *J* = 5.7 Hz, 1H), 7.21 – 7.41 (m, 4H), 7.52 – 7.59 (m, 2H), 7.73 (t, *J* = 8.2 Hz, 2H); ^{13}C NMR (100.67 MHz, $CDCl_3$) 26.3, 26.4 (CH_2), 27.7, 27.7 (CH_2), 28.1, 28.1 (CH_2), 28.4, 28.4 (CH_3), 28.4, 28.4 (CH_3), 28.4, 28.5 (CH_3), 29.7, 29.7 (CH_2), 40.4, 40.5 (CH), 47.2, 47.3 (CH_2), 48.4, 48.4 (CH_2), 48.6, 48.7 (CH_2), 67.4, 67.7(CH_2), 79.2, 79.3 (C), 119.9,

119.9 (CH), 119.9, 119.9 (CH), 124.8, 124.8 (CH), 124.8, 124.9 (CH), 125.0, 125.0 (CH), 127.1, 127.1 (CH), 127.6, 127.6 (CH), 127.7, 127.7 (CH), 141.2, 141.3 (C), 141.4, 141.4 (C), 143.9, 143.9 (C), 144.0, 144.0 (C), 156.0, 156.1 (C=O), 156.6, 156.7 (C=O), 173.7, 173.7 (C=O); HRMS (Waters Synapt G2): m/z calcd for $[C_{28}H_{36}N_2O_6 +H]^+$: 497.2652; Found: 497.2649.

General Procedure for the Solid Phase peptide Synthesis (SPPS) of **P1-P3**:

Deprotection of Fmoc- protecting:

A 20% (v/v) piperdine in DMF solution was added to a pre-swelled Rink amide resin ((50 mg; loading value: 0.70 mmol/g) and mixed well by means of N_2 bubbling for 15 min. Beads were collected by filtration, washed with DMF (3 x 4 mL), CH_2Cl_2 (2 x 4 mL) and DMF (2 x 5 mL). A small portion of these beads was checked by Kaiser Test. The whole process was repeated three times.

Peptoide coupling reaction:

To a suspension of above prepared resin (ca. 50 mg) in 2 mL of DMF was added a solution of monomer (4 eq.) in DMF, HOBT (4 eq), HBTU (4 eq), DIPEA (4 eq) and mixed well by bubbling nitrogen gas for 1.5 h. The resin was filtered off, washed with DMF (3 x 4 mL), CH_2Cl_2 (2 x 4 mL) and DMF (2 x 5 mL). The successful peptide coupling reaction was confirmed by Kaiser Test on a few beads.

Cleavage from the resin:

Peptoid was cleaved from the resin by using a cleavage solution of 95% TFA; 5% DCM. 5 mL cleavage solution was added to the resin and incubated for 2.5 h. The resin was filtered and the filtrate was concentrated *in vacuo* to obtain a residue. The required peptoid was isolated by precipitating by the addition of cooled diethylether. The white precipitate was redissolved in CH_3CN and subjected to HPLC purification using reverse phase C18-silica column.

Biological Experiments

Preparation of the competent cells:

E.coli cells were grown in LB media for overnight for 37 ^oC. An inoculum of 500 µL from this culture broth was added to 50 mL of LB broth. Cells were shaken at 37 ^oC (200 rpm) for 2-3 h. The optical density was measure at 600nm until the absorbance

value reaches 0.6-0.9 arbitrary units. Cells were pelleted by spinning at 6000g/4 °C/5 min. The supernatant was aspirated off and cells were washed with 5 mL of magnesium chloride (100 mM) and pelleted again on centrifuge at 6000g/4 °C/5 min. Cells were suspended again in 8 mL of ice cold calcium chloride solution, and then spun again at 6000g/4 °C/5 min. Competent cells for further experiments was obtained by keeping them under 100 mM calcium chloride solution and incubated at 0 ^{o}C for 12 h. Glycerol was added and stored at -70°C.

Bacterial Transformation:

After preparing the competent cells, foreign DNA was mixed gently with the competent cell. It was kept in ice for 30 min and then heat shock was given at 42 °C for 90 sec. At that time, cells become porous and plasmid DNA enters into the cell. The resulting suspension was ice cooled for 2-3 min and then revived for 1 h in the LB medium (1 mL) at 37 °C. It was then centrifuged for 1 min and 900 µL was discarded. The cell pellet was resuspended in 100 µL and it was then plated in LB + Ampicillin plate. Plates were kept overnight at 37 °C. The transparent white colonies appeared, picked up single colony and grown in LB broth, then proceeded for plasmid isolation.

Plasmid Isolation:

Single colonies were inoculated in test tube containing 3-5 mL of LB medium containing ampicillin and cultures were grown overnight at 37 °C in the orbital shaker. Cultures were centrifuged in microfuge tubes for 10 min at 8000 rpm. Supernatant was discarded and the cell pellet was resuspended in 200 µL of solution-1. 200 µL of solution-2 was added and mixed gently for 2 min. Then 200 µL of solution-3 was added. It was then centrifuged for 10 min at 12000rpm. A white precipitate was formed which was discarded, the supernatant was transferred to a fresh tube and passed through the spin column at 12000 rpm for 1 min and wash buffer was used to give a clean-up and the residual buffer was removed by another round of centrifugation. The column was then put in a fresh Eppendorf tube. Elution buffer (50 µL) was added and plasmid DNA was eluted. The yield of DNA was quantified by nano-drop spectrophotometer and also by the agarose gel electrophoresis.

Solution-1: 50 mM Tris PH 8.0 with HCl, 10 mM EDTA, 100 µg/mL Rnase A.

Solution-2: 200 mM NaOH, 1% SDS.

Solution-3: 3.0 M potassium acetate, PH 5.5 adjusts by Acetic Acid.

Cell culture & Transfection:

Cell culture refers to growing the cells in a favourable environment, we cultured breast cancer cell line MCF7. Dulbecco's Modified Eagle's Medium (DMEM) was used. Transfection is the process of transforming nucleic acids into the cells, generally referring to the Eukaryotic cells by using the Reagent Lipofectamine 2000 which is cationic liposome. After treatment with that reagent, it will form liposome kind of structures surrounded by, positive charges. Cell membrane is negatively charged and the liposome structure keeps plasmid inside. For 200 ng of plasmid DNA, we added 0.2-0.6 µL of lipofectamine and 100 µL of OPTIMEM and incubated for 4 h and removed the OPTIMEM and added peptoids with different concentrations (1 µM, 10 µM, 20 µM, 50 µM, and 100 µM) growth media DMEM to the wells and incubated for 48 h at 37 °C.

Cell viability assay: (MTT assay, to calculate IC_{50} value):

MCF7 cells were distributed into a 96 well plate (~6000 cells/well), grown up to 70% confluency and then treated with three peptoids with different concentrations (2 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 200 µM, 500 µM, 1000 µM). After 48 hrs of the incubation with the peptoids, cells were treated with 3-(4, 5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazolium bromide and incubated for 4 h at 37 ^{o}C in CO_2 incubator. After incubation, medium was decanted and formazan crystals were dissolved in 200 µL of DMSO, sample absorbance was measured at 570 nm with reference wavelength at 630 nm. Data was normalized with the untreated cells.

Dual Luciferase Assay:

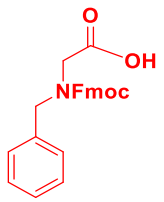
Dual Luciferase reporter assay system based on the two luciferase activities was performed. One reporter can be used to measure the response according to the experiment which is called as experimental reporter. The second reporter as an internal reporter to normalize the data obtained from the experimental reporter. The firefly luciferase from the firefly beetle is an enzyme which catalyses the oxidation of the luciferin that emits light at 560nm. Renilla luciferase from the sea pansy, oxidizes coelenterazine and emits light at 480nm. The Dual luciferase Assay activity of the firefly luciferase and the renilla luciferase are determined sequentially. Therefore in each well of the microplate, 100 µL of the firefly luciferase reagent

(LAR II) is added into the well containing the sample of cell lysate and the output of the light is measured over 10 secs. Then, 100 µL of second luciferase is injected to the well and again output is measured. The second reagent stops the first reaction and delivers the substrate of renilla luciferase reaction.

Conclusion

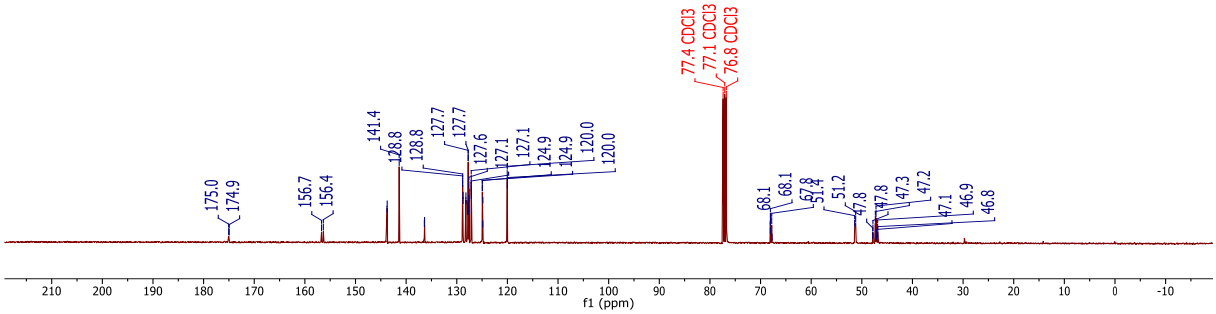
In conclusion, we have synthesized three peptoids which differ in the length of the side chain and the presence of guanidine residues by invoking solid phase peptide synthesis. Peptoids were synthesized on Rink amide resin and the peptoids were cleaved off from the resin by the addition of 95% TFA in CH_2Cl_2. Peptoids were purified using reverse phase C_{18}-silica column. Well purified peptoids were subjected to MTT and dual Luciferase assays to check the toxicity and miRNA-21 gene expression levels. Overall, the synthesized peptoids were found to have poor binding affinities at low peptoid concentrations. From the current experiments, it is not very clear whether compounds have entered the cell. In this regard, a fluorescent attached peptoids shall be synthesized and checked by FACS and confocal microscopy to investigate the cellular permeability. Subsequently, if the peptoids are permeable, then, new sequence of peptoids with more guanidine residues can be envisioned to have better biochemical profiles.

Spectral Chart

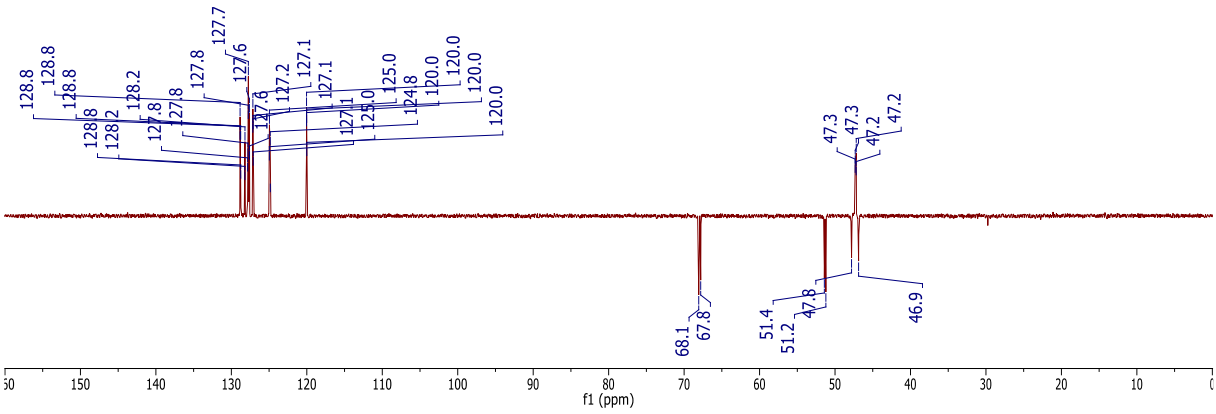


^{1}H NMR Spectrum (400.31 MHz, $CDCl_3$) of **M1**

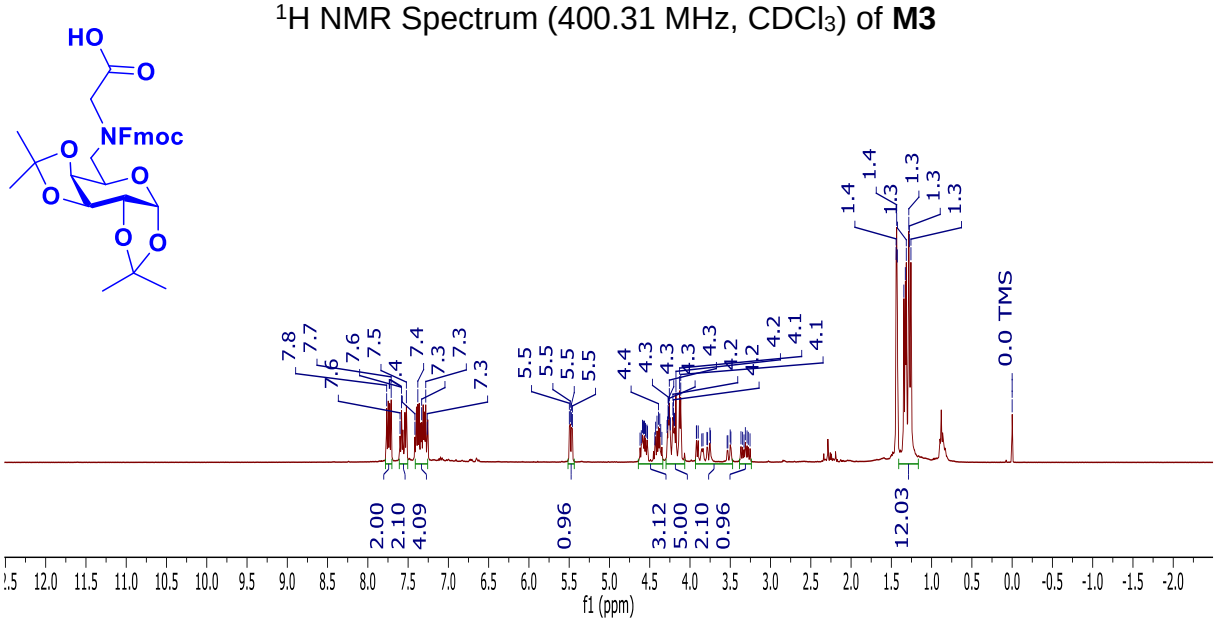
^{13}C NMR Spectrum (100.67 MHz, $CDCl_3$) of **M1**



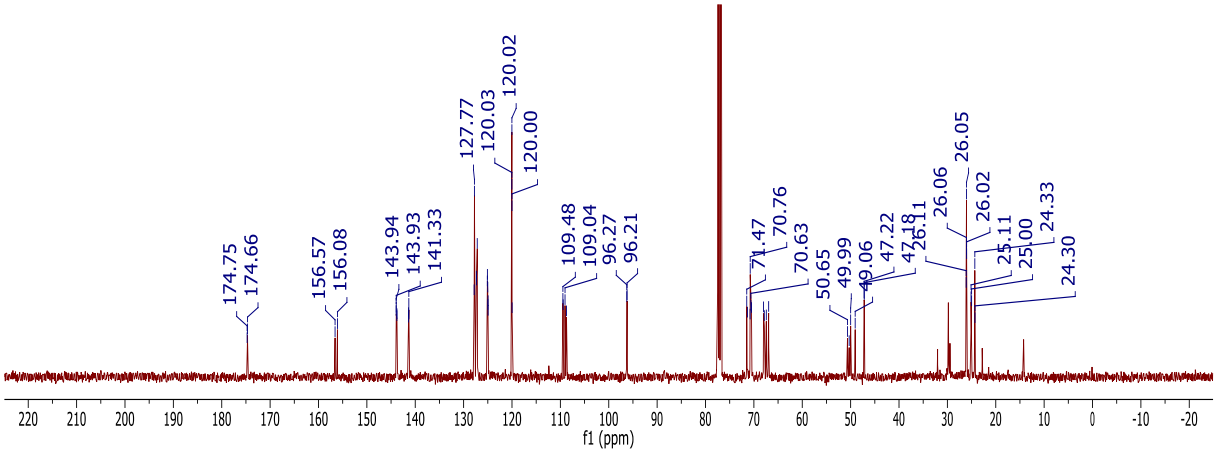
DEPT NMR Spectrum (100.67 MHz, $CDCl_3$) of **M1**



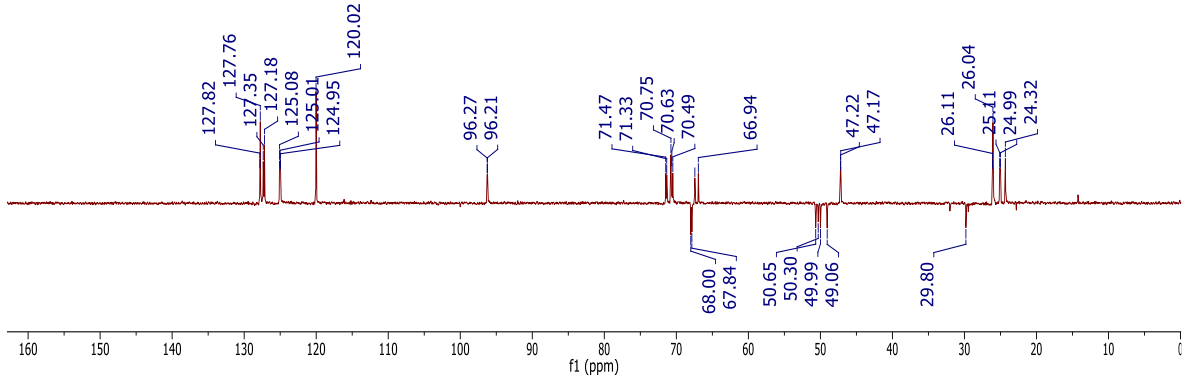
^{1}H NMR Spectrum (400.31 MHz, $CDCl_3$) of **M3**



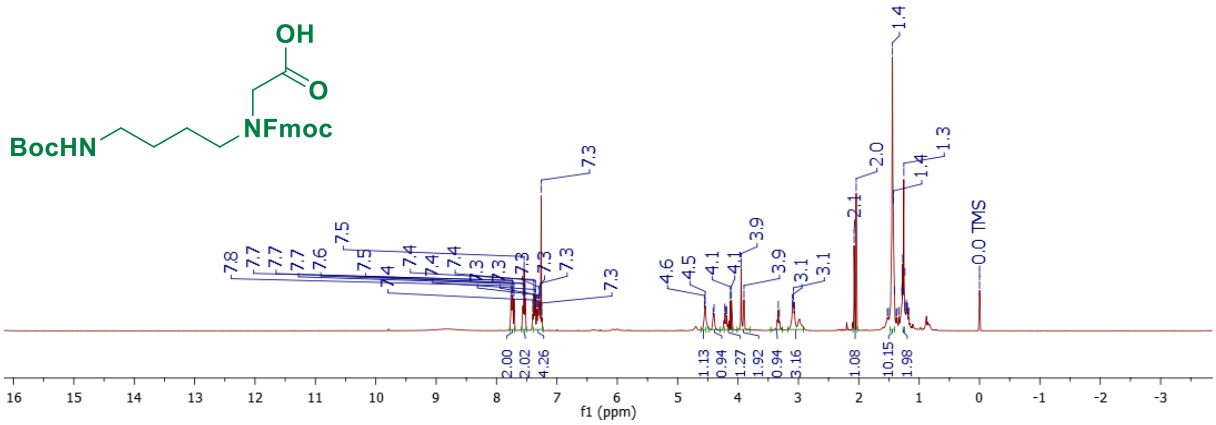
^{13}C NMR Spectrum (100.67 MHz, $CDCl_3$) of **M3**



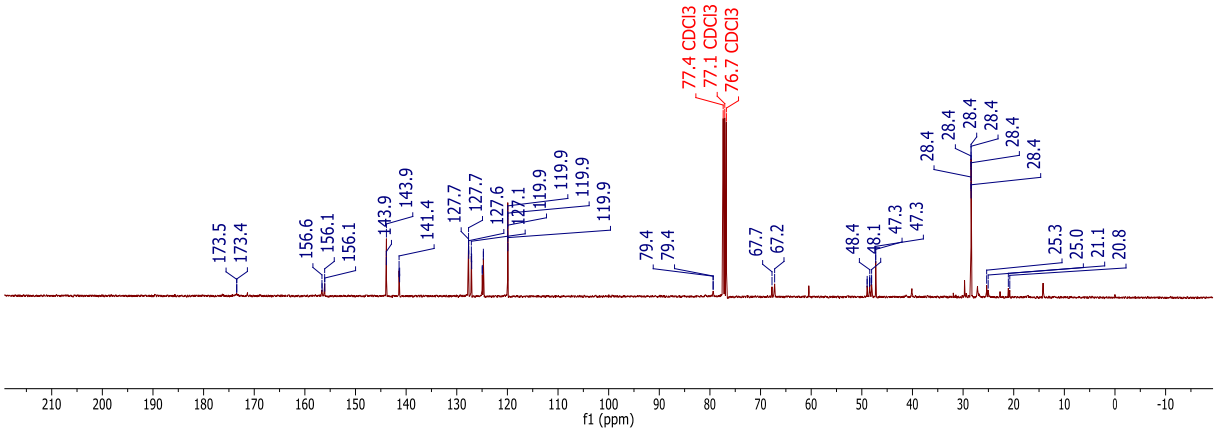
DEPT NMR Spectrum (100.67 MHz, $CDCl_3$) of **M3**



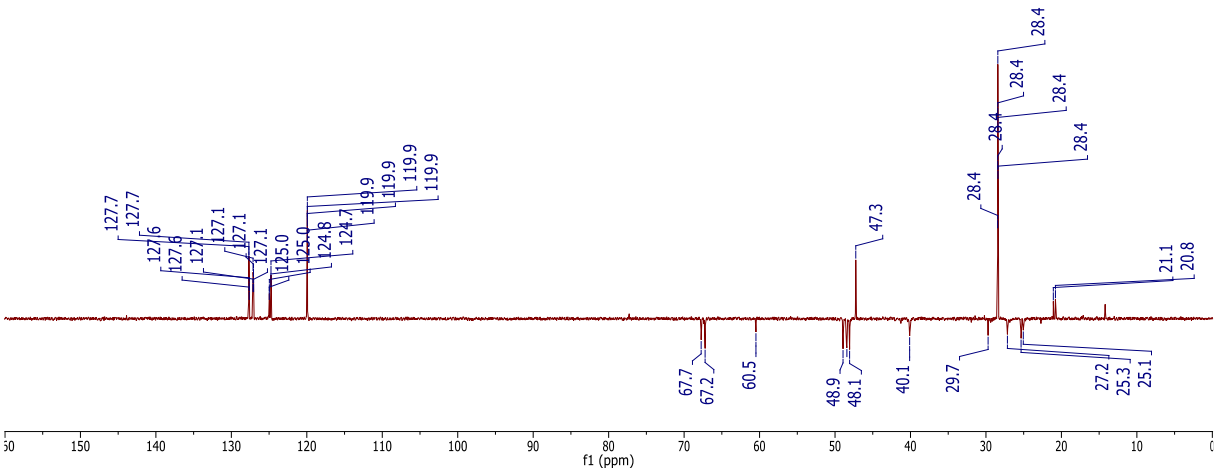
^{1}H NMR Spectrum (400.31 MHz, $CDCl_3$) of **M2**



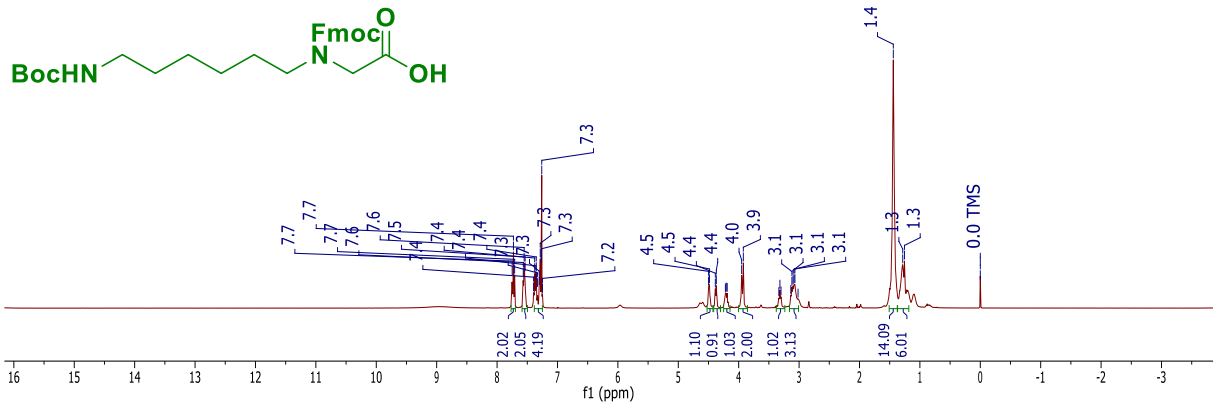
^{13}C NMR Spectrum (100.67 MHz, $CDCl_3$) of **M2**



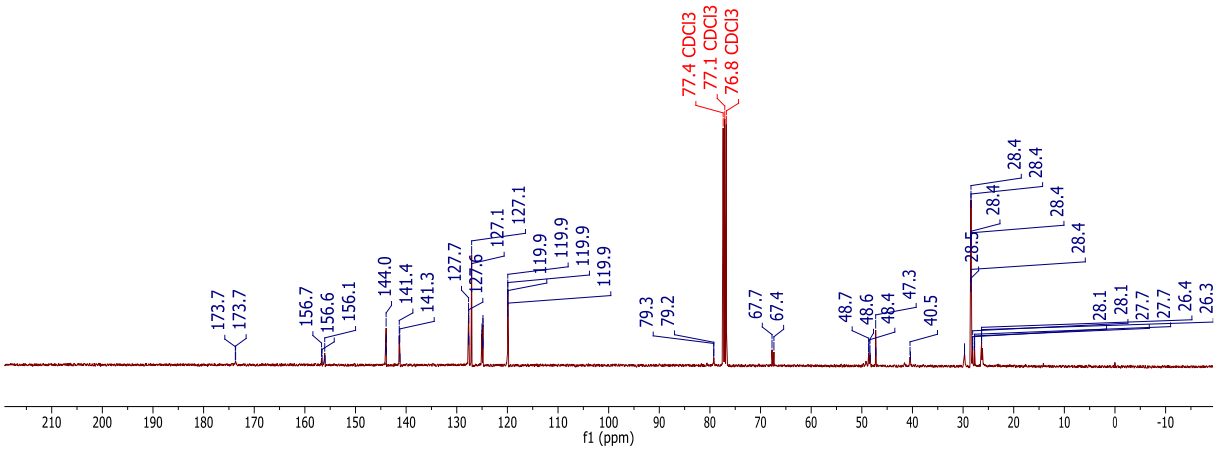
DEPT NMR Spectrum (100.67 MHz, $CDCl_3$) of **M2**



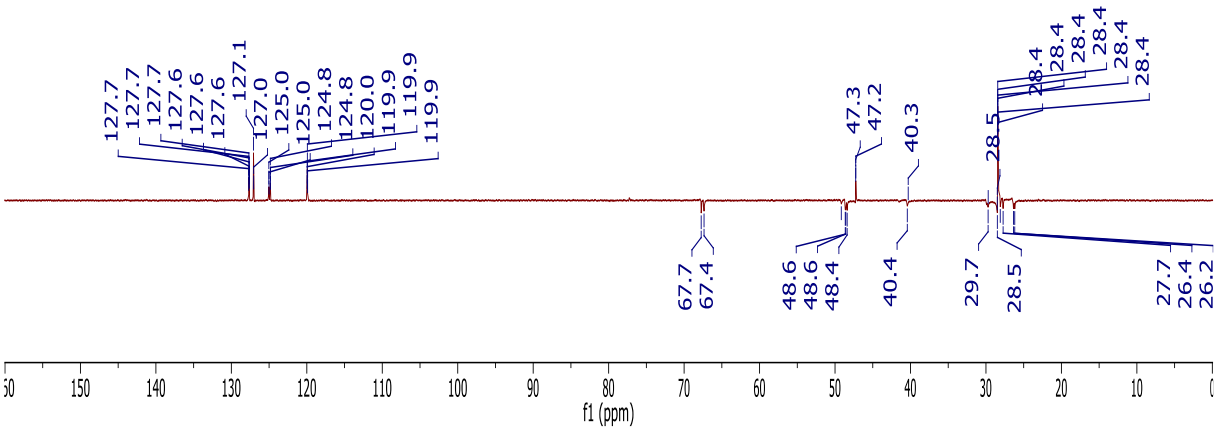
^{1}H NMR Spectrum (400.31 MHz, $CDCl_3$) of **M4**



^{13}C NMR Spectrum (100.67 MHz, $CDCl_3$) of **M4**



DEPT NMR Spectrum (100.67 MHz, $CDCl_3$) of **M4**

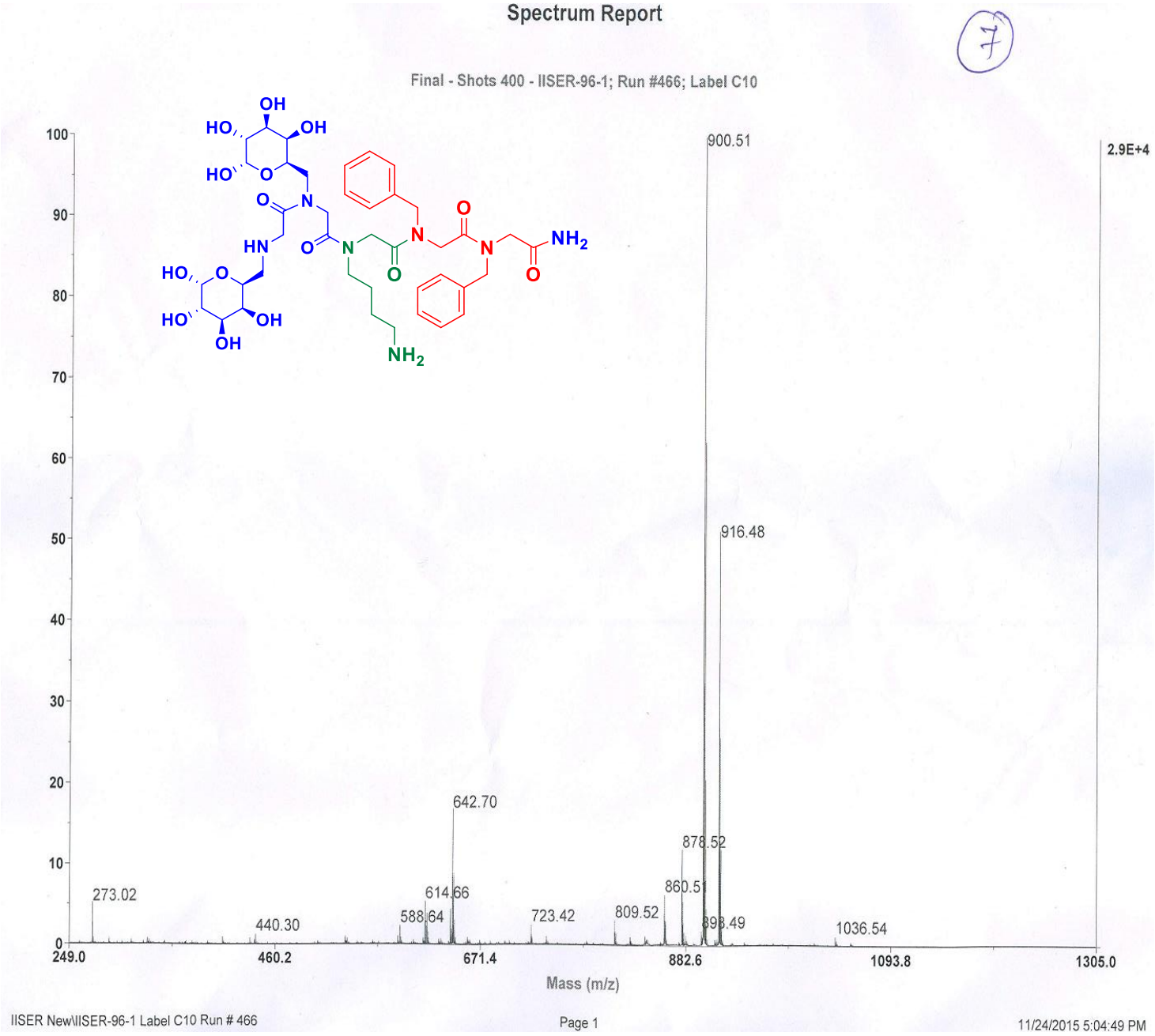


Peptoid-1 Confirmation by MALDI-TOF:

Expected m/z for Peptoid-1 (M+H$^+$): 878.41 Observed: 878.52

(M+Na): 900.39 Observed: 900.51

(M+K): 916.37 Observed: 916.48

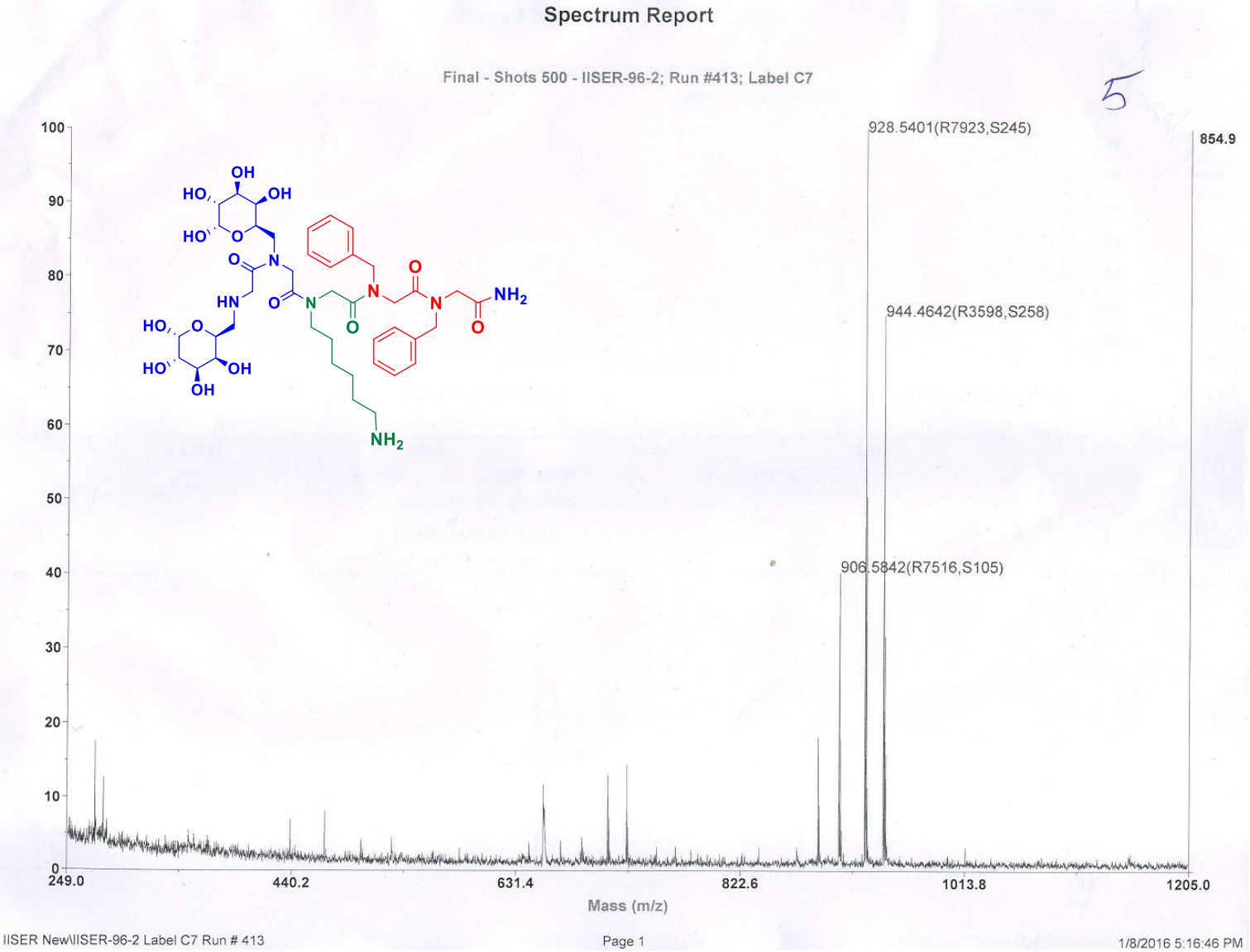


Peptoid-2 Conformation by MALDI-TOF:

Expected m/z for Peptoid-2 ($M+H^{+}$): 906.4460 Observed: 906.5842

(M+Na): 928.4280 Observed: 928.5401

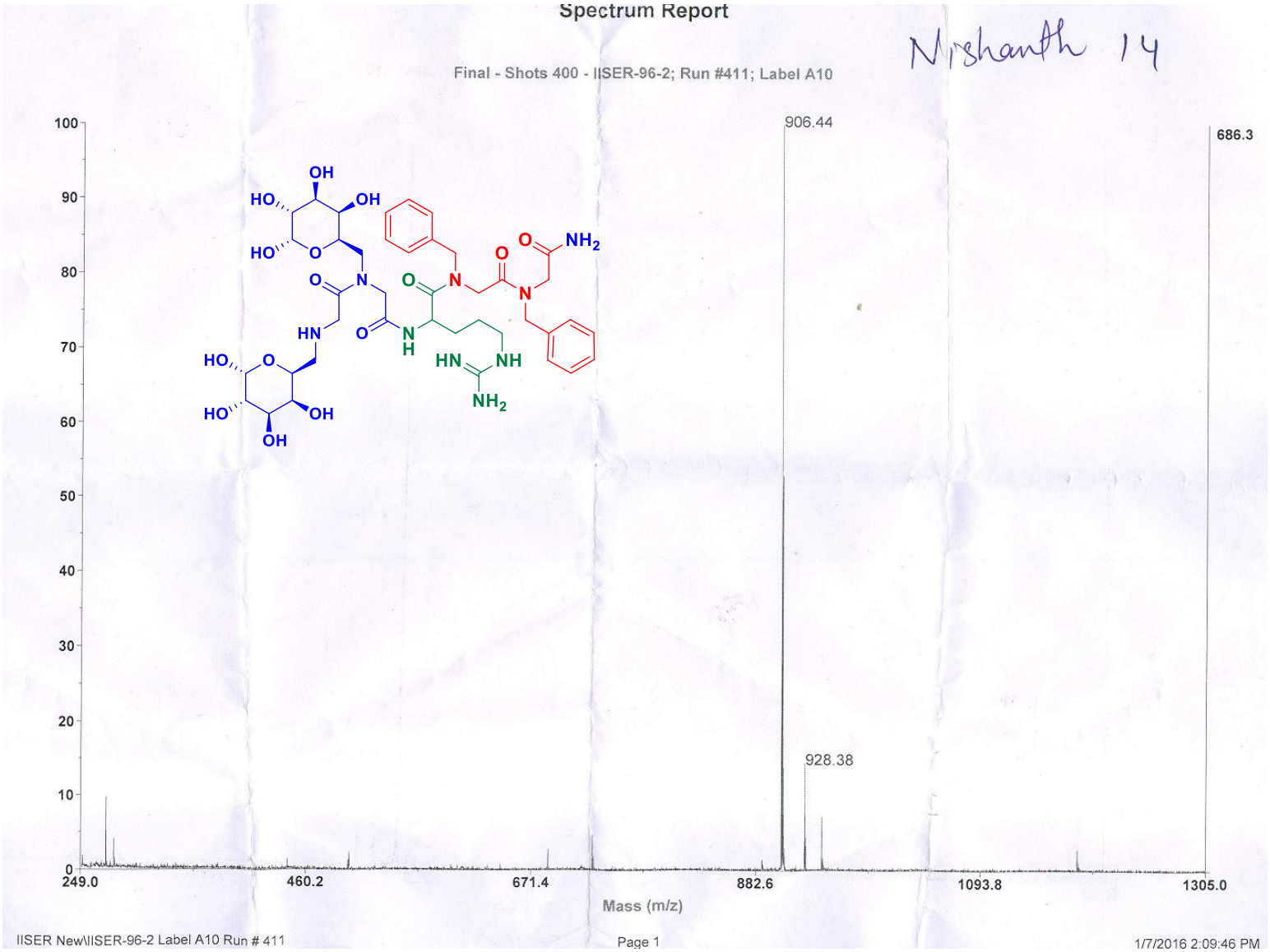
(M+K): 944.4019 Observed: 944.4642



Peptoid-3 Conformation by MALDI-TOF:

Expected m/z for Peptoid-2 $(M+H^{+})$: 906.42 Observed: 906.44

(M+Na): 928.40 Observed: 928.38



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