Rational Development of a Ligand for Theophylline Riboswitch Aptamer Expands Its Regulatory Dynamic Range in Prokaryotic and Eukaryotic Systems

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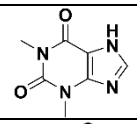
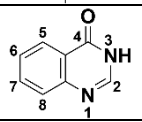
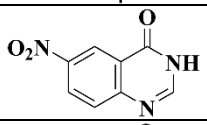
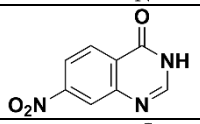
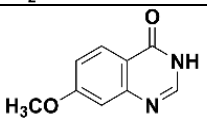
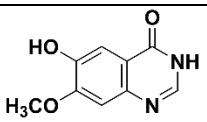
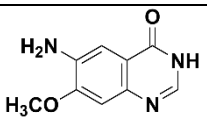
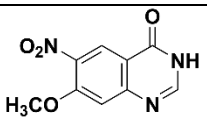
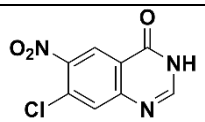
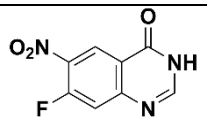
Table 1: Hits obtained from molecular docking of compound libraries with the theophylline aptamer (PDB ID: 8d28)

Sr. No.	Compound	Docking score
1	(2R,6S)-2-amino-7,9-dimethyl-1,2,3,6-tetrahydropurin-9-ium-6-ol	-11.23
2	6-fluoro-3-[2-(methylamino)ethyl]quinazolin-4-one	-10.70
3	1-cyclopentyl-3-[[2-(1,3-dioxo-4H-isoquinolin-2-yl)acetyl]amino]thiourea	-10.61
4	7-fluoro-3-[2-(methylamino)ethyl]quinazolin-4-one	-10.60
5	3-(aminomethyl)-8-methylquinazolin-4-one	-10.53
6	3-[2-(methylamino)ethyl]quinazolin-4-one	-10.53
7	3-(2-aminoethyl)quinazolin-4-one	-10.47
8	3-(aminomethyl)-2-chloro-6-fluoroquinazolin-4-one	-10.42
9	3-(aminomethyl)quinazolin-4-one	-10.13
10	1-methyl-7H-purin-1-ium-6-amine	-10.13
11	3-(aminomethyl)-7-fluoroquinazolin-4-one	-10.10
12	6-(3-aminopropyl)-4,9-dimethylpyrrolo[3,4-c]carbazole-1,3-dione	-10.09
13	3-(aminomethyl)-2-chloro-7-fluoroquinazolin-4-one	-10.08
14	5-(aminomethyl)-3H-quinazolin-4-one	-10.00
15	2-(6-carbamimidoyl-1H-benzimidazole-2-carbonyl)-3H-benzimidazole-5-carboximidamide	-9.98
16	6-fluoro-3-[3-(methylamino)propyl]quinazolin-4-one	-9.97
17	1,3-dimethylquinazolin-1-ium-4-one	-9.89
18	5,6-dimethoxy-2,3-dihydro-1H-inden-2-amine	-9.89
19	(2S)-2-amino-4-[[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl-methylamino]butanoic acid	-9.84
20	7-[2-hydroxy-3-[2-hydroxyethyl(methyl)amino]propyl]-1,3-dimethylpurine-2,6-dione	-9.84
21	6,7-dimethoxy-2-methyl-1,3-benzoxazin-4-one	-9.81
22	(1R,2S)-2-amino-1,2,3,4-tetrahydronaphthalen-1-ol	-9.77
23	(3R)-3-(1,2,3,4-tetrahydroisoquinolin-7-yloxymethyl)-2,3-dihydrothieno[2,3-f][1,4]oxazepin-5-amine	-9.77
24	3-[1-(methylamino)ethyl]quinazolin-4-one	-9.76
25	3-[1-(3-aminopropyl)indol-3-yl]-4-(1H-indol-3-yl)pyrrole-2,5-dione	-9.76

26	2-(2-aminoethylamino)-3H-quinazolin-4-one	-9.75
27	2-amino-5-(aminomethyl)-3,7-dihydropyrrolo[2,3-d]pyrimidin-4-one	-9.75
28	3-(aminomethyl)-2-chloroquinazolin-4-one	-9.75
29	7-fluoro-3-(methylaminomethyl)quinazolin-4-one	-9.64
30	N-[(1S)-2-amino-1-phenylethyl]-5-(1H-pyrrolo[2,3-b]pyridin-4-yl)thiophene-2-carboxamide	-9.64
31	Phthalocyanine	-9.63
32	9-amino-n-[3-(dimethylamino)propyl]acridine-4-carboxamide	-9.62
33	3-(2-aminoethyl)-2-chloroquinazolin-4-one	-9.61
34	2-[[2-ethyl-6-[4-[2-(3-hydroxyazetidin-1-yl)-2-oxoethyl]piperazin-1-yl]-8-methylimidazo[1,2-a]pyridin-3-yl]-methylamino]-4-(4-fluorophenyl)-1,3-thiazole-5-carbonitrile	-9.61
35	1-(2-aminoethyl)quinazolin-4-one	-9.59
36	6-(1-Amino-3-iminoprop-1-enyl)quinazolin-4-amine	-9.57
37	5-(2-aminoethylamino)-6-fluoro-3-(1H-pyrrol-2-yl)-1H-benzo[cd]indol-2-one	-9.57
38	12-methyl-1,4-diazatetracyclo[7.6.1.05,16.010,15]hexadeca-9(16),10(15),11,13-tetraene	-9.56
39	2-amino-5-fluoro-3H-quinazolin-4-one	-9.56
40	2-(4-hydroxy-3-phenyl-1H-pyrazol-5-yl)-3H-benzimidazole-5-carboximidamide	-9.55
41	1,3-bis(4-amino-2-methylquinolin-6-yl)urea	-9.52
42	6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-4-ol	-9.52
43	12-methyl-1,4-diazatetracyclo[7.6.1.05,16.010,15]hexadeca-9(16),10(15),11,13-tetraene	-9.52
44	2,6-diamino-8-(1H-imidazol-2-ylsulfanylmethyl)-3H-quinazolin-4-one	-9.51
45	1,3-bis[3-(4,5-dihydro-1H-imidazol-2-yl)phenyl]urea	-9.47
46	1-amino-3-methylquinazolin-1-ium-4-one	-9.46
47	[amino-[2-[6-[amino(azaniumylidene)methyl]-1H-benzimidazole-2-carbonyl]-3H-benzimidazol-5-yl]methylidene]azanium	-9.41
48	2-amino-6-(hydroxymethyl)-3H-pteridin-4-one	-9.41
49	5-fluoro-3-(methylaminomethyl)quinazolin-4-one	-9.39
50	(1S,2S)-2-amino-1,2,3,4-tetrahydronaphthalen-1-ol	-9.39

Table 2: Molecular docking of synthesized derivatives with theophylline aptamer (PDB ID: 8d28)

Sr. No.	Compound	Structure	Docking score	K_D (µM)
1	Theophylline		-9.05	3.34
2	4-(1H)-quinazolinone		-6.51	NB
3	6-nitroquinazolin-4(3H)-one		-8.36	NB
4	7-nitroquinazolin-4(3H)-one		-8.20	NB
5	7-methoxyquinazolin-4(3H)-one		-8.21	1.50
6	6-hydroxy-7-mrthoxyquinazoli-4(3H)-one		-8.25	0.98
7	6-amino-7-methoxyquinazoli-4(3H)-one		-8.24	2.18
8	7-methoxy-6-nitroquinazolin-4(3H)-one		-8.62	0.89
9	7-chloro-6-nitroquinazolin-4(3H)-one		-8.32	1.58
10	7-floro-6-nitroquinazolin-4(3H)-one		-4.11	3.47

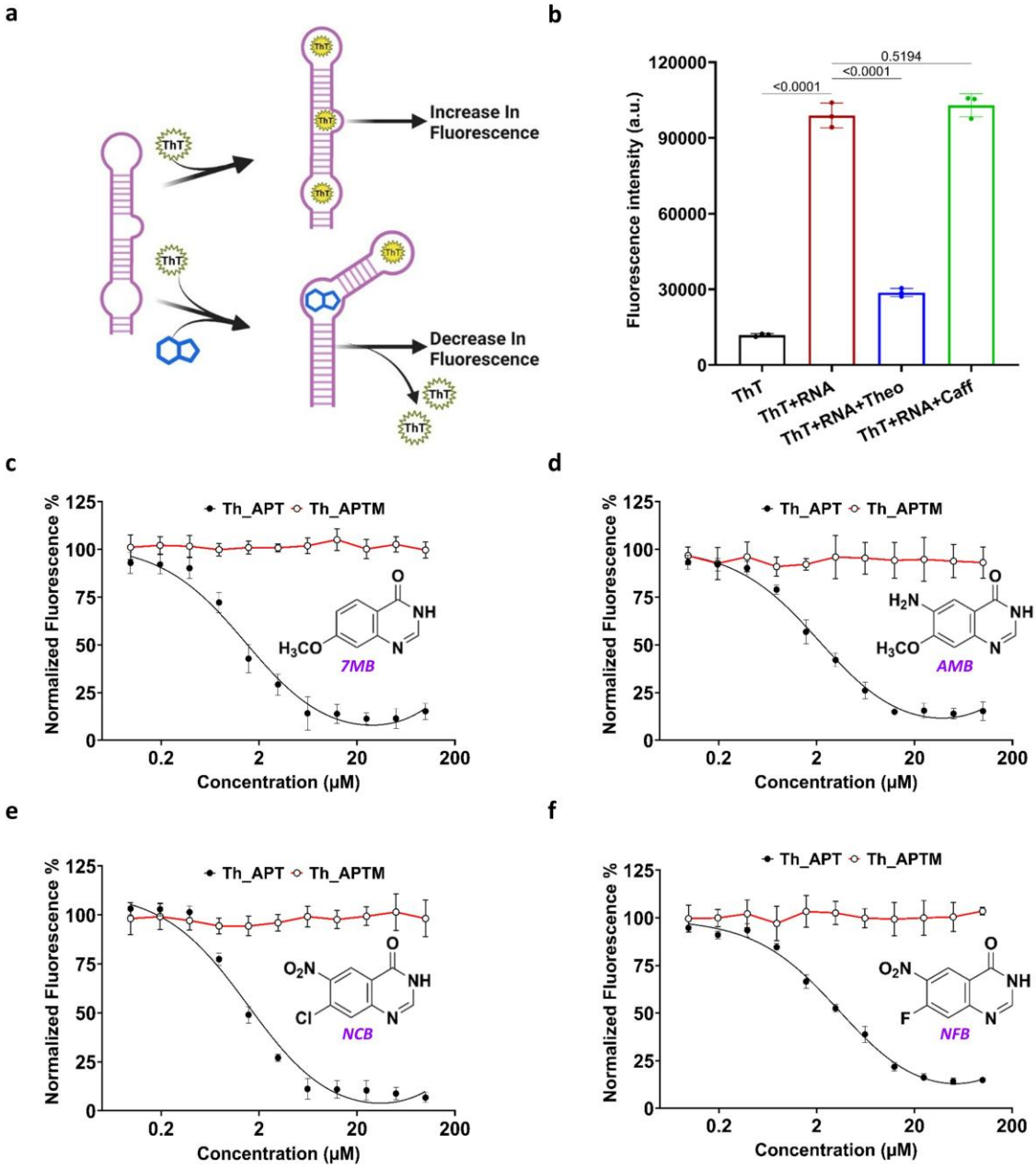


Figure S1 | ThT-based fluorescence assay for validation of aptamer ligand interactions: a, Illustration of the ThT-based fluorescent assay depicting the change in fluorescence in the presence and absence of the ligand. **b,** Change in fluorescence intensity of ThT in the presence of RNA, Theo, and Caffeine. Concentration-dependent change in relative fluorescence intensities of ThT at 25 °C after addition of ligand molecules **c,** 7MB. **d,** AMB. **e,** NCB. and **f.** NFB in a buffer containing 10 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, and 0.01 % Tween 20. Fluorescence intensity at 0 μM ligand concentration was considered as 100% and equilibrium dissociation constants (K_D) were determined using a one-site total and nonspecific binding equation in GraphPad Prism 10.4.2. (n = 3).

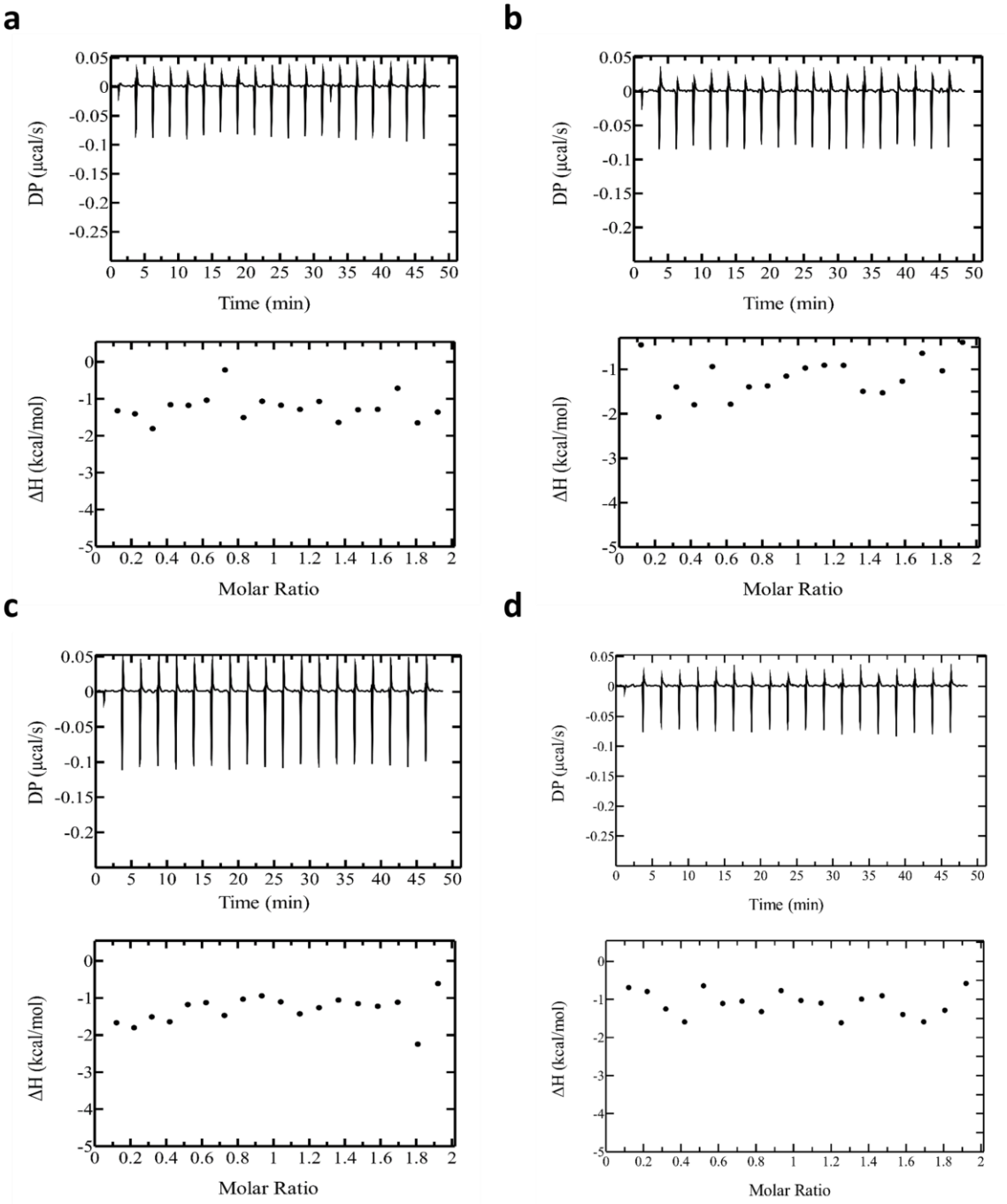


Figure S2 | Isothermal colorimetry with mutant aptamer – Th_APTM: Representative thermograms for ligands **a,** Theo, **b,** HMB, **c,** NMB, and **d,** Caff. With Th_APTM The upper panel displays the titration data, showing the heat released with each injection of ligand. The lower panel presents the integrated heat values, adjusted for the heat of dilution. Binding experiments were conducted at 25 °C in a buffer containing 10 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, and 1 % DMSO. The aptamer concentration in the cell was 5 µM, and the ligand in the syringe was at 50 µM.

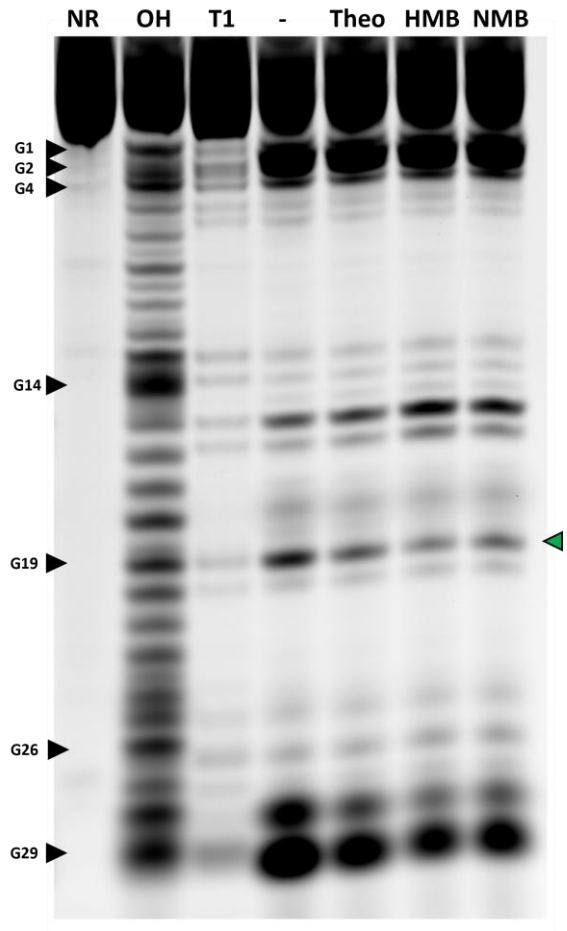


Figure S3 | RNase T1 footprinting of Cy5 labelled Th_APT: NR representes no reaction control, OH represents partially digested Th_APT with alkali, while T1 designates RNase T1 mediated cleavage of Th_APT under denaturing conditions. Lane (-), Theo, HMB and NMB rpresentes footprint of RNA oligonucleotide in the presence of DMSO, Theo, HMB and NMB.

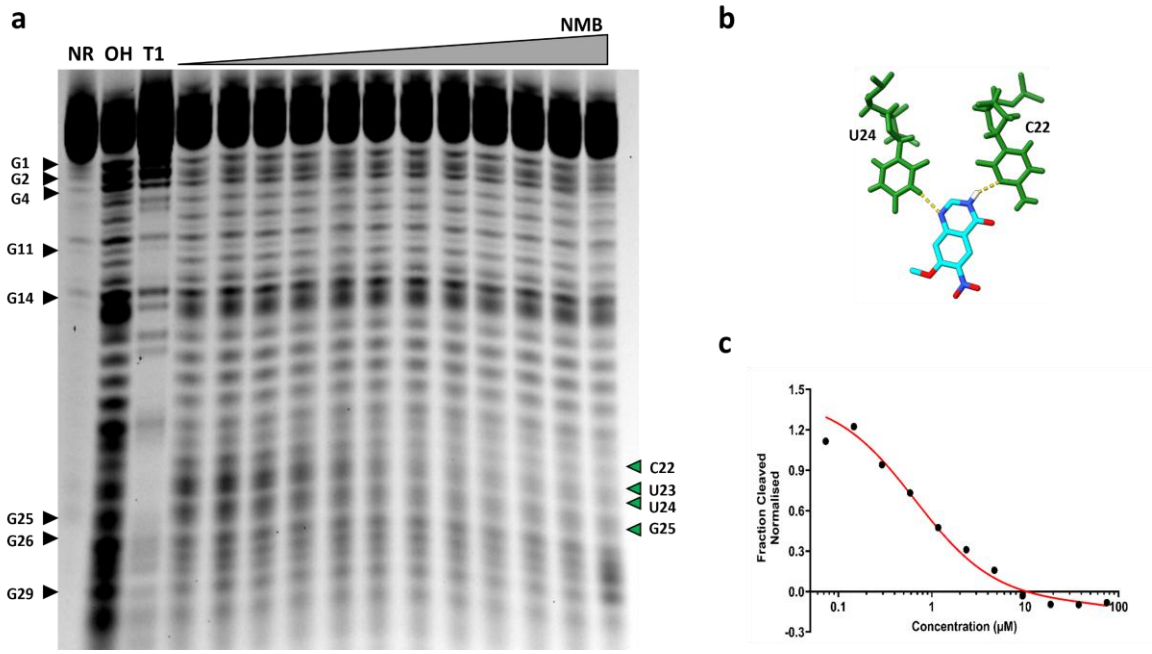


Figure S4 | Structural probing of ligand-induced conformational changes: a, Inline probing gel image of lanes loaded with RNA Th_APT subjected to no reaction (NR), alkaline pH (OH), RNase T1 (T1), or reactions with various concentrations of NMB. **b,** molecular interactions of ligand molecule NMB with nucleotides C22 and U24. **c,** The plot of fraction of RNA cleaved versus molar concentration of ligand NMB. Fraction cleaved values at nucleotide position C22 were estimated using ImageQuant^{TM} on the extent of band intensities.

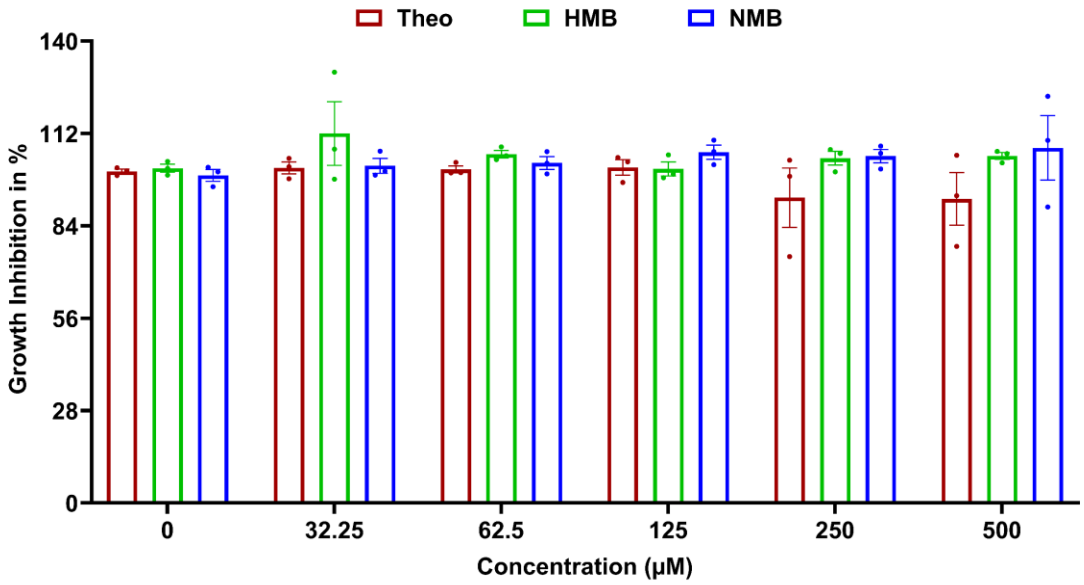


Figure S5 | Effect of ligand molecules on bacterial growth: *E. coli* DH10β cells were treated with ligand molecules Theo, HMB, and NMB. Data are represented as a percentage of bacterial growth, with the growth of *E. coli* without ligand treatment set as 100% control (n=3 data represented as mean ± SEM).

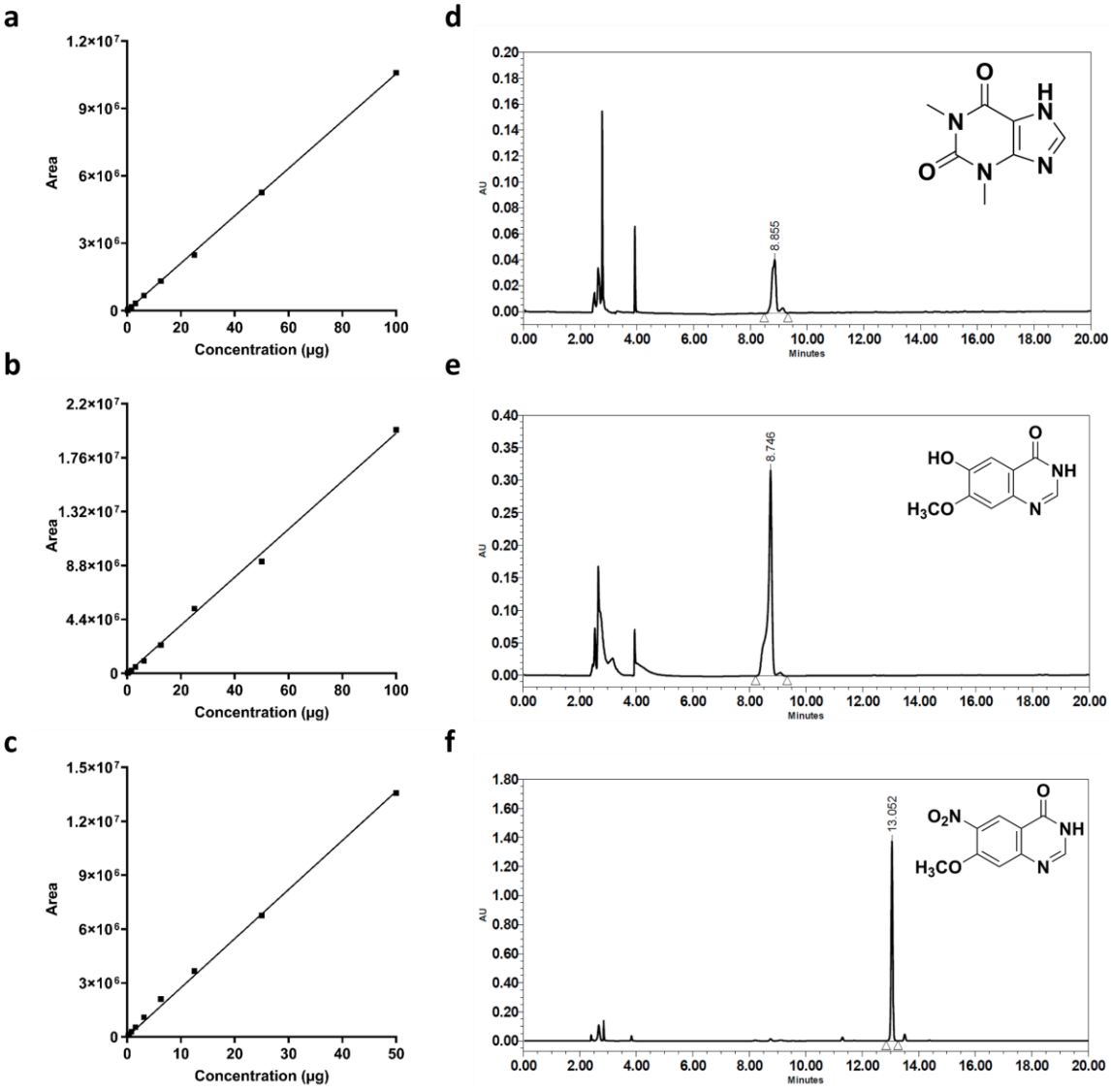


Figure S6 | Estimation of intracellular ligand concentrations in *E. coli* DH10β: Standard graph of concentration versus area for **a,** Theo. **b,** HMB. and **c,** NMB. HPLC chromatograms obtained at 280 nm for extracts obtained from bacterial lysate upon treatment with **d**, Theo. **e**, HMB and, **f**, NMB (n=3).

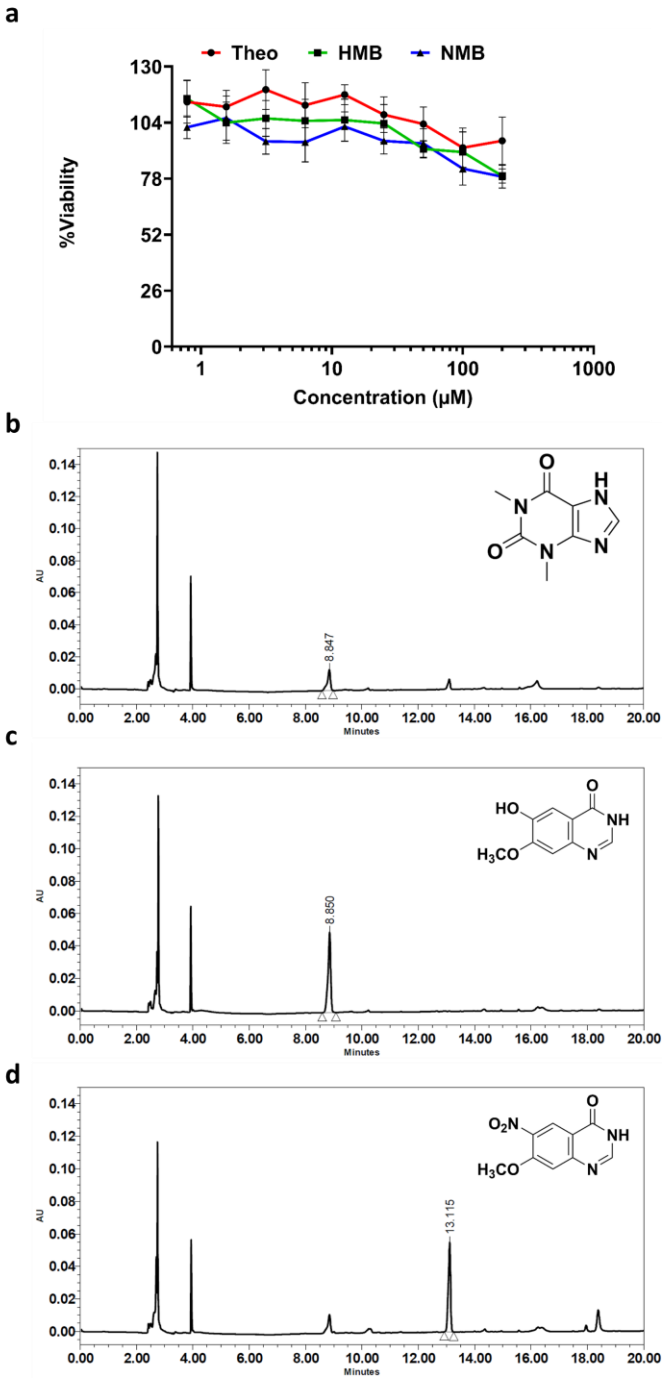


Figure S7 | Analysis of cell cytotoxicity and intracellular ligand concentration in HEK-293T cells: **a,** Effect of ligand molecules on cell survival in HEK-293T cells. HPLC chromatograms obtained at 280 nm for extracts from cell lysate upon treatment with **b**, Theo. **c**, HMB and, **d**, NMB (n=3).

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RS-1       TCTAGAACG AGTCCAGAAG TTCAC----- -GGAGATACC AGCATCGTCT TGATGCCCTT GGCAGCTCCT CCCTACTAAA GGAGGTCATC CGATG
RS-2       TCTAGAACG AGTCCAGAAG TTCACGGTAC CGGAGATACC AGCATCGTCT TGATGCCCTT GGCAGCTCCC TGCT----AA GGAGGCAACA AGATG
RS-3       TCTAGAACG AGTCCAGAAG TTCAC----- -GGAGATACC AGCATCGTCT TGATGCCCTT GGCAGCTCCC TGCT----AA GGAGGCAACA AGATG
RS-4       TCTAGAACG AGTCCAGAAG TTCACGGTAC CGGTGATACC AGCATCGTCT TGATGCCCTT GGCAGCACCC TGCT----AA GGAGGCAACA AGATG
RS-5       TCTAGAACG AGTCCAGAAG TTCAC----- -GGTGATACC AGCATCGTCT TGATGCCCTT GGCAGCACCC TGCT----AA GGAGGCAACA AGATG
RS-6       TCTAGAACG AGTCCAGAAG TTCAC----- -GGAGATACC AGCATCGTCT TGATGCCCTT GGCAGCTCCT CCCTACT-AA GGAGG-CATC CGATG
RS-7       TCTAGAACG AGTCCAGAAG TTCACGGTAC CGGAGATACC AGCATCGTCT TGATGCCCTT GGCAGCTCCT CCCTACTAAA GGAGGTCATC CGATG
RS-8       TCTAGAACG AGTCCAGAAG TTCACGGTAC CGGAGATACC AGCATCGTCT TGATGCCCTT GGCAGCTCCT CCCTACT-AA GGAGG-CATC CGATG
RS-9       TCTAGAACG AGTCCAGAAG TTCAC----- -GGTGATACC AGCATCGTCT TGATGCCCTT GGCAGCACCT CCCTACTAAA GGAGGTCATC CGATG
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Figure S8 | Sequence alignment for riboswitch constructs with varying pre and post aptameric regions for riboswitch constructs RS-1 to RS-9.

Table 3: Activation ratios obtained for different riboswitches:

Sr. No.	Riboswitch	Theo	HMB	NMB
1	RS-1	4.91	16.12	12.74
2	RS-2	13.81	24.98	21.84
3	RS-3	35.75	74.98	88.09
4	RS-4	32.78	112.04	109.21
5	RS-5	22.25	47.06	45.11
6	RS-6	7.89	17.36	16.28
7	RS-7	5.85	14.66	10.35
8	RS-8	1.62	4.55	4.75
9	RS-9	1.39	4.66	5.10

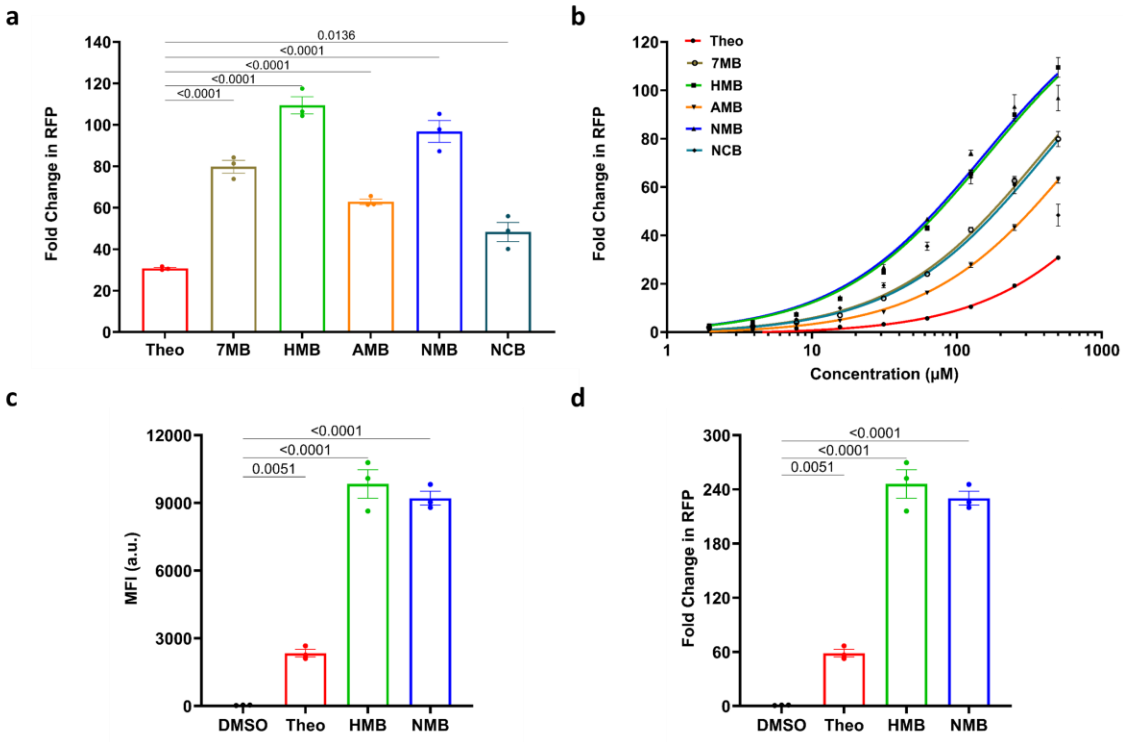

Figure S9 | Activity of ligands with RS-4 construct in *E. coli* DH10β cells: a, Fold increase in expression of RFP for RS-4 upon treatment with 500 μM of ligand (Theo, 7MB, HMB, AMB, NMB, and NCB). **b,** Dose-dependent increase in the fold change of RFP expression for RS-4 across a ligand concentration range of 0 - 500 μM. EC_{50} was calculated using the dose–response stimulation model (log[agonist] vs. response) in GraphPad Prism 10.4.2. **c,** Median fluorescence (MFI) values obtained from FACS analysis for RS-4 upon treatment with ligand molecules. **d,** Fold change in RFP expression obtained from MFI values in FACS analysis (n=3).

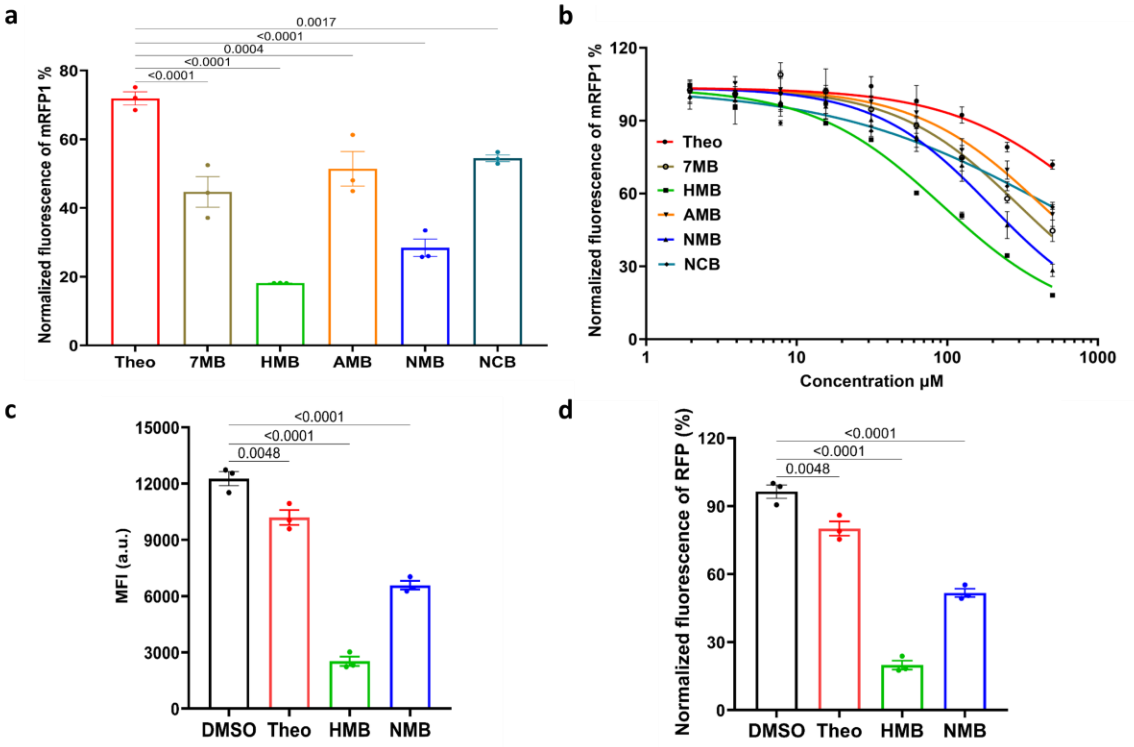

Figure S10 | Activity of ligands with RS-10 construct in *E. coli* DH10β cells: a, % decrease in expression of RFP for RS-10 upon treatment with 500 µM of ligand (Theo, 7MB, HMB, AMB, NMB, and NCB). **b,** Dose-dependent decrease in RFP expression for RS-10 across a ligand concentration range of 0 - 500 µM. EC_{50} was calculated using the dose–response stimulation model (log[agonist] vs. response) in GraphPad Prism 10.4.2. **c,** Median fluorescence (MFI) values obtained from FACS analysis for RS-10 upon treatment with ligand molecules. **d,** Fold change in RFP expression obtained from MFI values in FACS analysis (n=3)

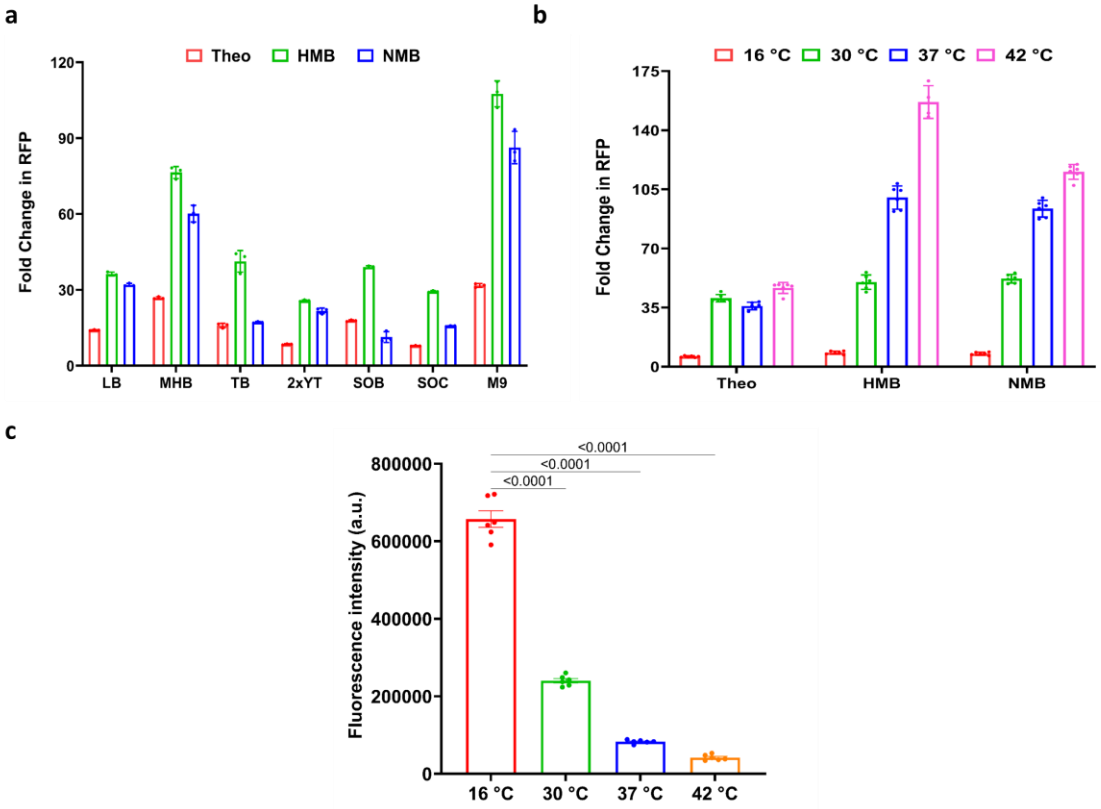

Figure S11 | Characterization of activity of ligands in different physiological conditions with RS-4: a, Effect of different media compositions on riboswitch-mediated gene regulation in the presence of different ligand molecules. **b,** Effect on expression of RFP in the presence of different ligands under different growth temperatures. **c,** Fluorescence of RFP in the presence of DMSO at different temperatures (n=3).

Table 4: Primers used to synthesize templates used in cloning to generate various riboswitch constructs with different pre- and post-aptameric regions.

Sr. No.	Construct	Oligonucleotide sequence
1	RS-2	CGCCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGTGATA CCAGCATCGTCTTGATGCCCTTGGCAGCACCCTGCTAAGGAG GCAACAAGATGCGTCGTCGCACCGCGAGCTCGCG RS-2_Fw: CGCCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGTGATA CCAGCATCGTCTTGATGCCCTTGGCAGCACCCTGC RS-2_ Rv: CGCGAGCTCGCGGTGCGACGACGCATCTTGTTGCCTCCTTAG CAGGGTGCTGCCAAGG
2	RS-3	CGCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGAGATAC CAGCATCGTCTTGATGCCCTTGGCAGCTCCTCCCTACTAAAG GAGGTCATCCGATGCGTCGTCGCACCGCGAGCTCCGC RS-3_Fw: TAATACGACTCACTATAGGGGAGCGTTCAGCATCGTCTTGAT GCC RS-3_Rv: GGACCAGACAAGGGCATCAAGACGATGCTG
3	RS-4	CGCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGAGATAC CAGCATCGTCTTGATGCCCTTGGCAGCTCCTCCCTACTAAGG AGGCATCCGATGCGTCGTCGCACCGCGAGCTCCGC RS-4_Fw: CGCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGAGATAC CAGCATCGTCTTGATGCCCTTGGCAGCTCCTCCCTAC RS-4_Rv: GCGGAGCTCGCGGTGCGACGACGCATCGGATGCCTCCTTAG TAGGGAGGAGCTGCCAAGGG
4	RS-5	CGCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGAGATAC CAGCATCGTCTTGATGCCCTTGGCAGCTCCCTGCTAAGGAGG CAACAAGATGCGTCGTCGCACCGCGAGCTCCGC RS-5_Fw: CGCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGAGATAC CAGCATCGTCTTGATGCCCTTGGCAGCTCCCTGC RS-5_Rv: GCGGAGCTCGCGGTGCGACGACGCATCTTGTTGCCTCCTTAG CAGGGAGCTGCCAAGG

5	RS-6	CGCTCTAGAACGAGTCCAGAAGTTCACGGAGATACCAGCAT CGTCTTGATGCCCTTGGCAGCTCCCTGCTAAGGAGGCAACAA GATGCGTCGTCGCACCGCGAGCTCCGC RS-6_Fw: CGCTCTAGAACGAGTCCAGAAGTTCACGGAGATACCAGCAT CGTCTTGATGCCCTTGGCAGCTCCCTGCTAAGG RS-6_Rv: GCGGAGCTCGCGGTGCGACGACGCATCTTGTTGCCTCCTTAG CAGGGAGCTGC
6	RS-7	CGCTCTAGAACGAGTCCAGAAGTTCACGGTGATACCAGCAT CGTCTTGATGCCCTTGGCAGCACCCTGCTAAGGAGGCAACA AGATGCGTCGTCGCACCGCGAGCTCCGC RS-7_Fw: CGCTCTAGAACGAGTCCAGAAGTTCACGGTGATACCAGCAT CGTCTTGATGCCCTTGGC RS-7_Rv: GCGGAGCTCGCGGTGCGACGACGCATCTTGTTGCCTCCTTAG CAGGGTGCTGCCAAGGGCATCAAGAC
7	RS-8	CGCTCTAGAACGAGTCCAGAAGTTCACGGTGATACCAGCAT CGTCTTGATGCCCTTGGCAGCACCTCCCTACTAAAGGAGGTC ATCCGATGCGTCGTCGCACCGCGAGCTCCGC RS-8_Fw: CGCTCTAGAACGAGTCCAGAAGTTCACGGTGATACCAGCAT CGTCTTGATGCCCTTGGC RS-8_Rv: GCGGAGCTCGCGGTGCGACGACGCATCGGATGACCTCCTTT AGTAGGGAGGTGCTGCCAAGGGCATCAAGACG
8	RS-9	CGCTCTAGAACGAGTCCAGAAGTTCACGGTGATACCAGCAT CGTCTTGATGCCCTTGGCAGCACCTCCCTACTAAGGAGGCAT CCGATGCGTCGTCGCACCGCGAGCTCCGC RS-9_Fw: CGCTCTAGAACGAGTCCAGAAGTTCACGGTGATACCAGCAT CGTCTTGATGCCCTTGGC RS-9_Rv: GCGGAGCTCGCGGTGCGACGACGCATCGGATGCCTCCTTAG TAGGGAGGTGCTGCCAAGGGCATCAAGAC
9	RS-10	TCTAGAACGAGTCCAGAAGTTCACGGTACCGGGATACCAGC CGAAAGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTTATC GAATTAAGGAGGCAACAAGATGCGTCGTCGCACCGCGAGCT C

		RS-10_Fw: CGCTCTAGAACGAGTCCAGAAGTTCACGGTACCGGGATACC AGCCGAAAGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTT ATCG RS-10_Rv: GCGGAGCTCGCGGTGCGACGACGCATCTTGTTGCCTCCTTAA TTCGATAACCGGGTCGCATC
10	RS-1_M	TCTAGAACGAGTCCAGAAGTTCACGGAGCGTTCAGCATCGT CTTGATGCCCTTGTCTGGTCCTCCCTACTAAAGGAGGTCATC CGATGCGTCGTCGCACCGCGAGCTC RS-1_M_Fw: GATGCCCTTGTCTGGTCCTCCCTAC RS-1_M_Rv: AAGACGATGCTGAACGCT

Table 5: Primers used to synthesize templates used in IVT for ITC

Sr. No.	Construct Name/code	Oligonucleotide sequence
1	Th_Apt	**TAATACGACTCACTATAGG**GGAGATACCAGCATCG TCTTGATGCCCTTGGCAGCTCC Aeb_TTase A Fw: TAATACGACTCACTATAGGGGAGATACCAGCATCG Aeb_TTase A Rv: GGAGCTGCCAAGGGCATCAAGACGATGCTGGTATCTC CCC
2	Th_APTM	**TAATACGACTCACTATAGG**GGAGCGTTCAGCATCGTC TTGATGCCCTTGTCTGGTCC Aeb_Des_SP Fw: TAATACGACTCACTATAGGGGAGCGTTCAGCATCGTCT TGATGCC Aeb_53 Rv: GGACCAGACAAGGGCATCAAGACGATGCTG

Table 6: Primers used to synthesize templates used in IVT for In-line probing, and T1-digestion

Sr. No.	Construct Name/code	Oligonucleotide sequence
1	Th_Apt	**TAATACGACTCACTATAGG**GGCGATACCAGCCGAAA GGCCCTTGGCAGCGTC Aeb_TTase A Fw: TAATACGACTCACTATAGGGGCGATACCAG Aeb_TTase A Rv: GACGCTGCCAAGGGCCTTTCGGCTGGTATCGCCCCTAT AG

Table 7: Primers used to synthesize templates used in IVT for the SPR experiment

Sr. No.	Construct Name/code	Oligonucleotide sequence
1	Th_APT_SP	**TAATACGACTCACTATAGG**GGAGATACCAGCATCGTCTTGATGCCCTTGGCAGCTCCAAAAAAAAAAAAAAAAAAAAAAAA Aeb_TTase A Fw: TAATACGACTCACTATAGGGGAGATACCAGCATCG Aeb_SP_A Rv: TTTTTTTTTTTTTTTTTTTTTTGGAGCTGCCAAGGGCATCAAGACGATGCTGGTATCTCCCC
2	Th_APTM_SP	**TAATACGACTCACTATAGG**GGAGCGTTCAGCATCGTCTTGATGCCCTTGGCAGCTCCAAAAAAAAAAAAAAAAAAAAAAAA Aeb_DES_SP Fw: TAATACGACTCACTATAGGGGAGCGTTCAGCATCGTCTTGATGCC Aeb_DES_SP Rv: TTTTTTTTTTTTTTTTTTTTTTGGAGCTGCCAAGGGCATCAAGACGATGCTGAACGCTCCCC

Table 8: DNA primers used in qRT-PCR

Sr. No.	Construct Name/code	Oligonucleotide sequence
1	RFP 1	CGTATGTACCCGGAAGACGG
2	RFP 2	GTTTTGTAAGCACCCGGCAG
3	GAPDH 1	CTCACCGGATGCACCAATGTT
4	GAPDH 2	CGCGTTGCTCACAATGTTCAT
5	m-Cherry 1	GTCCTCGAAGTTCATCACGC
6	m-Cherry 2	TTCATGTACGGCTCCAAGGC
7	BFP 1	CAAGACCACATATAGATCC
8	BFP 2	TGGCCTCCTTGATTCTTTCC

Sequencing Data for all the riboswitch constructs used in this study

RS 2

CAACGAAGCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTT
AGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCT
TATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCGC
CAGATATCCTCGAGCGCGGAATTCCCTAGGGGATCCGTTGACGGCTAGCTCAGTCCT
AGGTACAGTGCTAGCTTCTAGAACGAGTCCAGAAGTTCACGGTACCGGTGATACCA
GCATCGTCTTGATGCCCTTGGCAGCACCCTGCTAAGGAGGCAACAAGATGCGTCGT
CGCACCGCGAGCTCTGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTATG
GAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTCC
GTACGAAGGTACCCAGACCGCTAAACTGAAAGTTACCAAAGGTGGTCCGCTGCCGT
TCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGTTAAAC
ACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGG
AACGTGTTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTCCC
TGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCG
ACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCACCGAACGTATG
TA

RS 3

CAAACGGATGCAATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCA
TTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTG
ATCTTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTT
TCGCCAGATATCCTCGAGCGCGGAATTCCCTAGGGGATCCGTTGACGGCTAGCTCAG
TCCTAGGTACAGTGCTAGCTTCTAGAACGAGTCCAGAAGTTCACGGTACCGGAGATA
CCAGCATCGTCTTGATGCCCTTGGCAGCTCCTCCCTACTAAAGGAGGTCATCCGATG
CGTCGTCGCACCGCGAGCTCTGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTT
CGTATGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGG
TCGTCCGTACGAAGGTACCCAGACCGCTAAACTGAAAGTTACCAAAGGTGGTCCGC
TGCCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACG
TTAAACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCA
AATGGGAACGTGTTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACT
CCTCCCTGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCC
CGTCCGACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCACCGAA
CGTATGTA

RS 4

CATTGGAAGCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTT
TAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATC
TTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCG
CCAGATATCCTCGAGCGCGGAATTCCCTAGGGGATCCGTTGACGGCTAGCTCAGTCC
TAGGTACAGTGCTAGCTTCTAGAACGAGTCCAGAAGTTCACGGTACCGGAGATACC
AGCATCGTCTTGATGCCCTTGGCAGCTCCTCCCTACTAAGGAGGCATCCGATGCGTC

GTCGCACCGCGAGCTCTGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTA
TGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGT
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GTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGTTAA
ACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATG
GGAACGTGTTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTC
CCTGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTC
CGACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCACCGAACGTA
TGTA

RS 5

GCTCCGGATGCCATTGGGGAATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCAT
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ACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATG
GGAACGTGTTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTC
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CCGACGGTCCGGTTATGCAAAAAAAAAACCATGGGTTGGGAAGCTTCCCACCGAAC
GTATGTA

RS 6

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TAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATC
TTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCG
CCAGATATCCTCGAGCGCGGAATTCCCTAGGGGATCCGTTGACGGCTAGCTCAGTCC
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TGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGGAACGTGT
TATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTCCCTGCAAGA
CGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTCC
GGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTTCCACCGAACGTATGTA

RS 7

CATCGATCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTTA
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ATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCGCC
AGATATCCTCGAGCGCGGAATTCCCTAGGGGATCCGTTGACGGCTAGCTCAGTCCTA
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ACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGTTAAACACCCGGCTG
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TGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTCCCTGCAAGACG
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RS 8

CTTCGAAGGCCATTGGGAATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTT
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ATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCGCC
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TTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTCCCTGCAAG
ACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTC
CGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCACCGAACGTATGTA

RS 9

CTTCGAAGCATTGGGAATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTTA
GCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTT
ATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCGCC
AGATATCCTCGAGCGCGGAATTCCCTAGGGGATCCGTTGACGGCTAGCTCAGTCCTA
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GGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTCCG
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RS 10

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TCTTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTT
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ACCAACTTCCCGTCCGACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGC
TTCCACCGAACGTATGTA

RS 1_M

CTACACGAAGGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCC
ATTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGT
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TTCGCCAGATATCCTCGAGCGCGGAATTCCCTAGGGGATCCGTTGACGGCTAGCTCA
GTCCTAGGTACAGTGCTAGCTTCTAGAACGAGTCCAGAAGTTCACGGAGCGTTCAGC
ATCGTCTTGATGCCCTTGTCTGGTCCTCCCTACTAAAGGAGGTCATCCGATGCGTCG
TCGCACCGCGAGCTCTGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTAT
GGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTC
CGTACGAAGGTACCCAGACCGCTAAACTGAAAGTTACCAAAGGTGGTCCGCTGCCG
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GACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCACCGAACGTAT
GTA

Promoter

Riboswitch

Ribosomal Binding Site

Start Codon

Red Fluorescent Protein

General materials and synthetic procedures

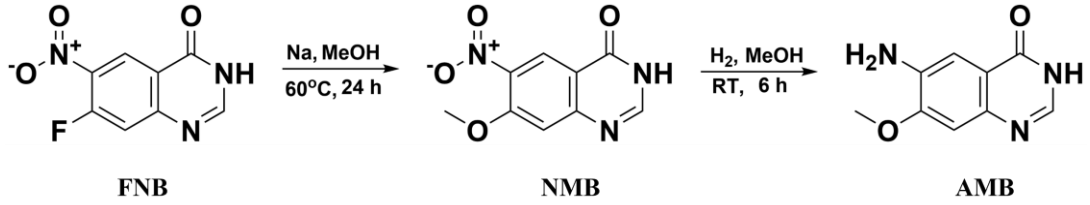
Synthesis and Characterization of 4-(1H)-quinazolinone derivatives:

The compounds were synthesized in the laboratory. All the standard chemicals and solvents were purchased from commercial suppliers (Sigma, SRL, TCI, and Fisher Scientific). Silica gel plates were used for thin-layer chromatography (TLC), and the plates were visualized with UV light. Silica gel (Kieselgel 60, Merck, 0.063 – 0.200 mm) was used for column chromatography. NMR spectra were acquired on a Bruker Avance III HD 300 and 400 spectrometer.

Synthesis of 7-methoxy-6-nitroquinazolin-4(3H)-one (NMB):

To synthesize compound NMB (7-methoxy-6-nitroquinazolin-4(3H)-one) (**Scheme 1**), commercially available starting material, i.e. FNB (7-fluoro-6-nitroquinazolin-4(3H)-one) (Cas no 162012-69-3) (1 eq.), was dissolved in 100 mL methanol, and sodium powder (3eq.) was added and stirred at 60 °C for 24 hours. Subsequently, the reaction mixture was dried and extracted using DCM. Finally, it was purified using flash chromatography using 5 % Methanol in DCM and characterized using ^{1}H & ^{13}C NMR.

NMR characterization of 7-methoxy-6-nitroquinazolin-4(3H)-one (NMB): ^{1}H NMR (300 MHz, DMSO-d6 ppm) 4.04 (s, 3H), 7.42 (s, 1H), 8.22 (s, 1H), 8.51 (s, 1H), 12.66 (s, 1H). **^{13}C NMR** (75 MHz DMSO-d6) 57.83, 111.00, 115.67, 124.23, 138.72, 149.27, 153.60, 156.4,5 159.98.

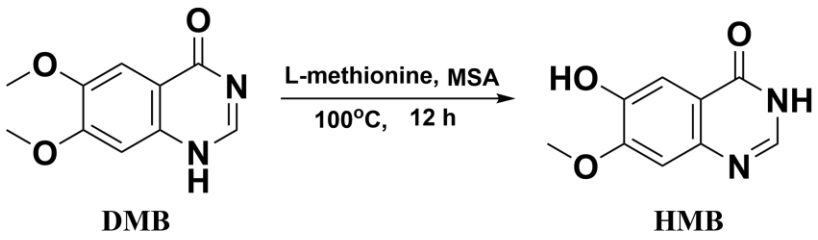


Scheme 1. Synthetic scheme of NMB & AMB

Synthesis of 6-amino-7-methoxyquinazolin-4(3H)-one (AMB):

Compound 6-amino-7-methoxyquinazolin-4(3H)-one (AMB) (**Scheme 1**) is synthesized by reducing the nitro group of NMB (7-methoxy-6-nitroquinazolin-4(3H)-one) to amine in the presence of a reducing environment (H_2 gas) in methanol at room temperature for 6 hours. Reaction was monitored through the course of time in DCM-methanol 5 %. Finally, after completion of the reaction, the compound is purified using flash chromatography and characterized using 1H NMR and 13C NMR.

NMR characterization of 6-amino-7-methoxyquinazolin-4(3H)-one (AMB): ^{1}H NMR (300 MHz, DMSO-d6 ppm) 3.91 (S, 3H), 5.31 (S, 2H), 6.99 (S, 1H), 7.23 (S, 1H), 7.80 (S, 1H), 11.78 (S, 1H). **^{13}C NMR** (75 MHz DMSO-d6) 56.21, 106.20, 107.07, 117.09, 138.60, 141.74, 142.09, 152.60, 160.60.



DMB HMB

Scheme 2. Synthetic scheme of HMB

Synthesis of 6-Hydroxy-7-methoxy-3H-quinazolin-4-one (HMB):

Compound HMB was synthesized as represented in Scheme 2. To synthesize compound HMB (6-Hydroxy-7-methoxy-3H-quinazolin-4-one), commercially available starting material compound DMB (6,7-Dimethoxy-1H-quinazolin-4-one) (Cas no 13794-72-4) (3.06 g) was diluted with 20mL of methanesulfonic acid. 2.66 g of L-methionine was added to the resulting solution & stirred at 100◦C for 12 hours. Ice was added to the reaction and neutralized with 40 % aq. NaOH to induce crystallization of the product. The solid was filtered under reduced pressure, washed with water, and air-dried to obtain the title compound (2.67 g, 94 %).

NMR characterization of 6-Hydroxy-7-methoxy-3H-quinazolin-4-one (HMB): ^{1}H NMR (400 MHz, DMSO-d6 ppm) 3.90 (S, 3H), 7.09 (S, 1H), 7.38 (S, 1H), 7.91 (S, 1H), 9.82 (S, 1H), 11.92 (S, 1H) **^{13}CNMR** (100 MHz DMSO-d6) 56.29, 108.64, 109.15, 116.49, 143.43, 144.27, 147.03, 154.35, 160.50.

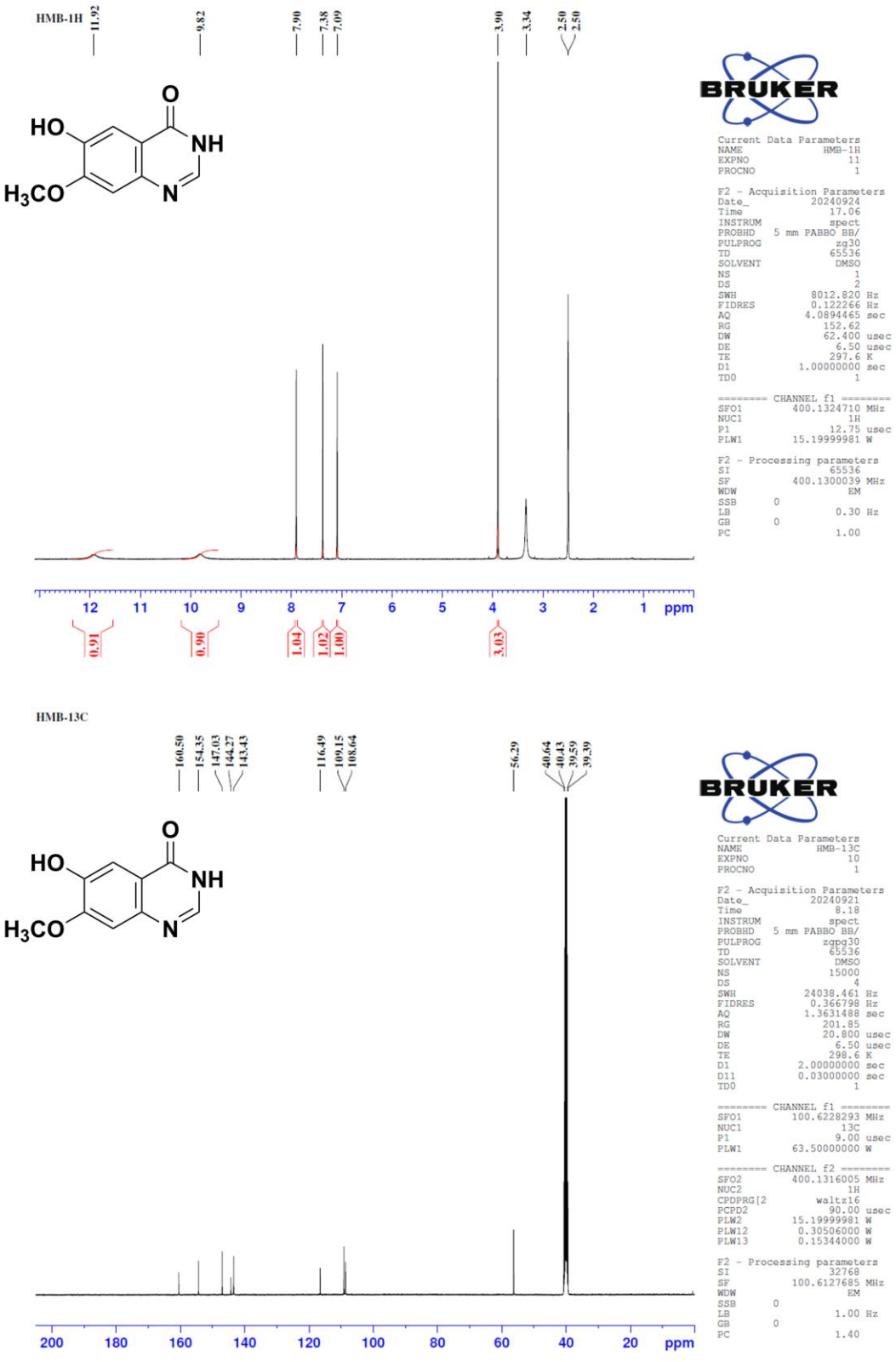


Figure S12 | ^{1}H NMR and ^{13}C NMR data of HMB

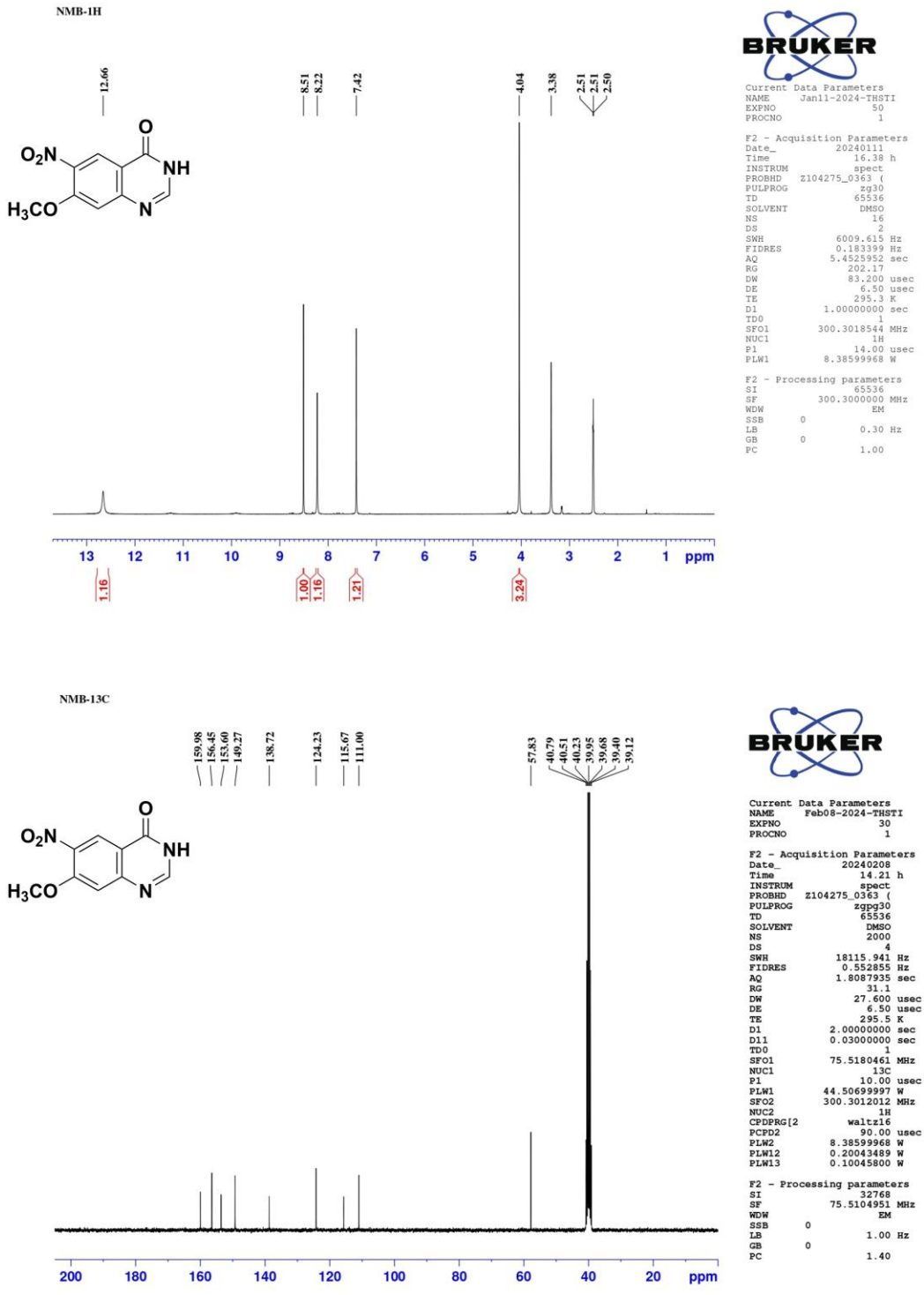


Figure S13 | ^{1}H NMR and ^{13}C NMR data of NMB

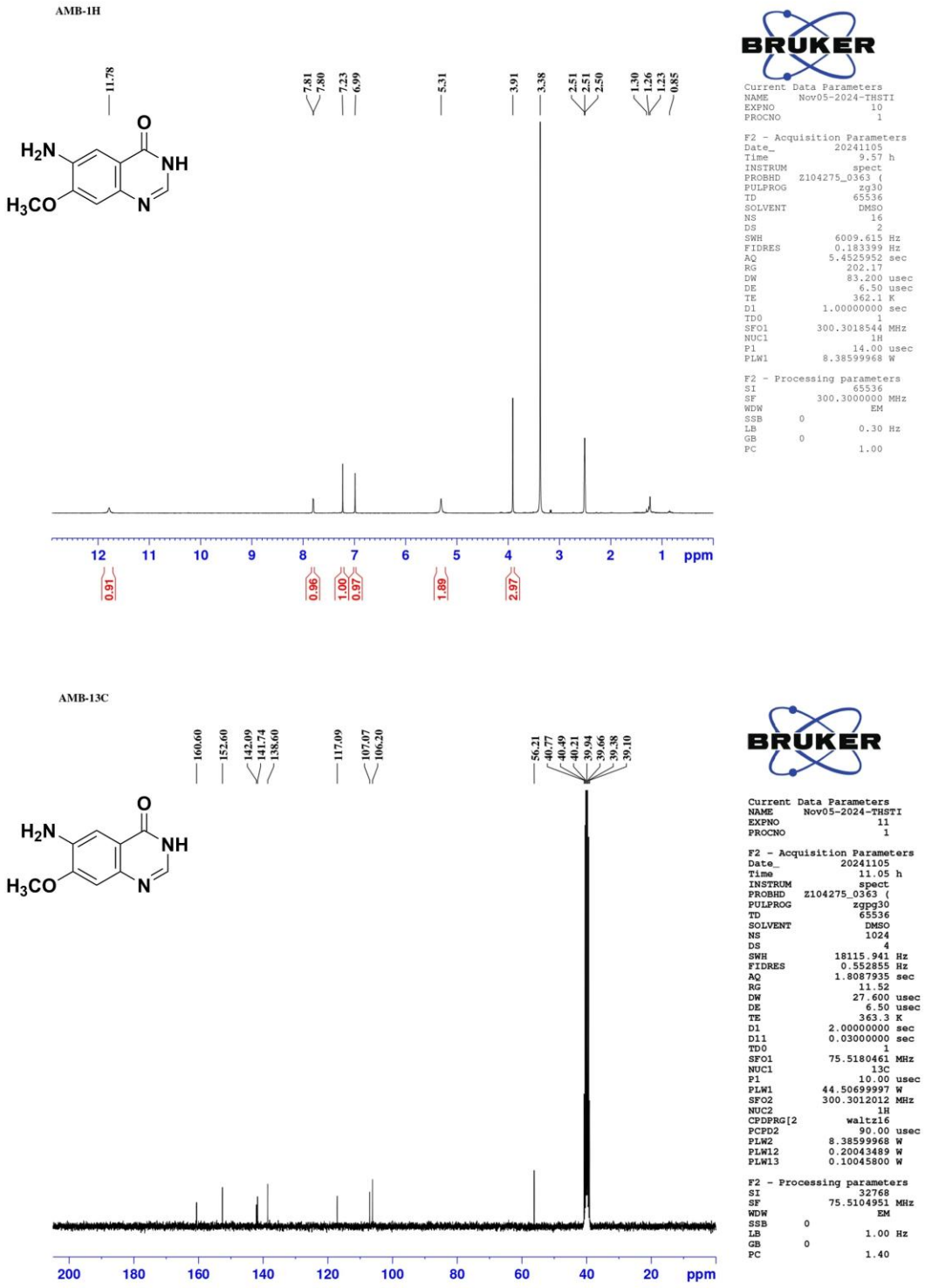


Figure S14 | ^{1}H NMR and ^{13}C NMR data of AMB

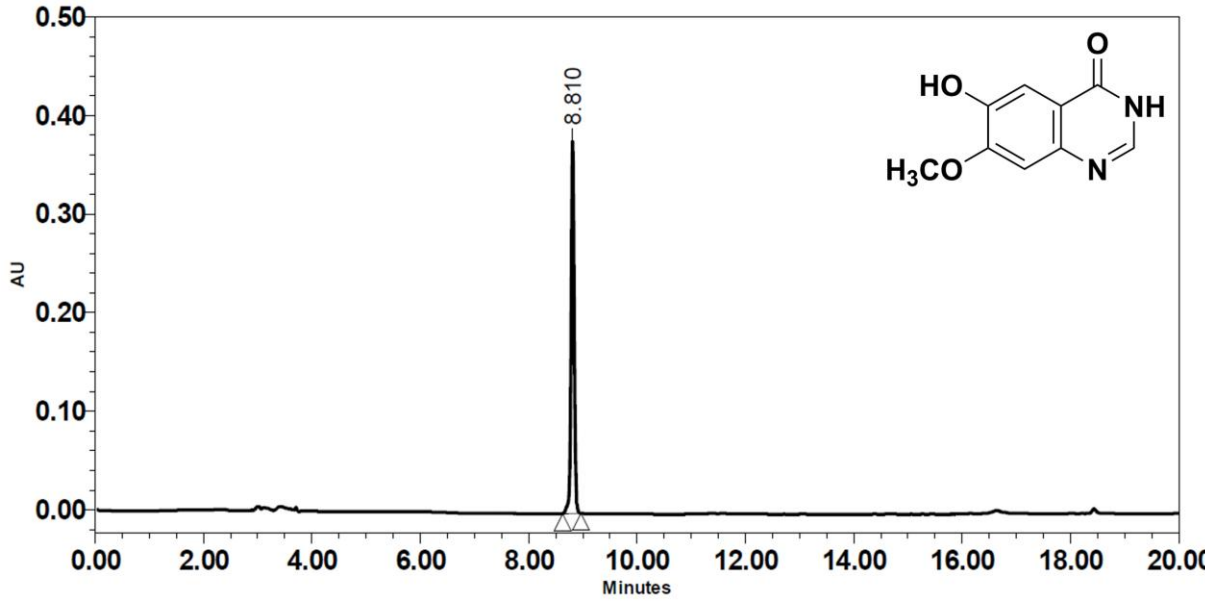


Figure S15 | HPLC Chromatogram of HMB at 280 nm

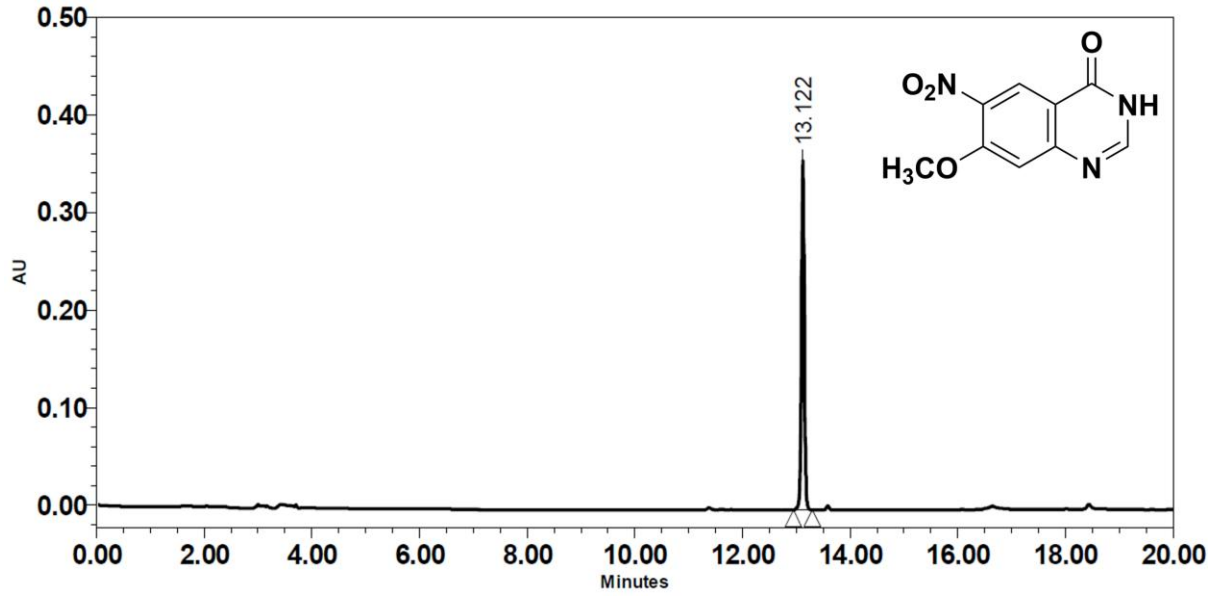


Figure S16 | HPLC Chromatogram of NMB at 280 nm