Supplementary Information

Identification of morpholine based hydroxylamine analogues: Selective inhibitors of MARK4/Par-1d causing cancer cell death through apoptosis

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Synthesis Protocols

Protocol used for the synthesis of Arylaldoximes (9-16)

Hydroxylamine hydrochloride ($NH_4OH.HCl$, 75.0 mmol) was taken in 30 mL of water and stirred at 0 ºC. Afterwards 3N (75.0 mmol) of NaOH was added to this solution and then allowed to stir for 15 minutes at the same temperature. Different arylaldehydes (67.5 mmol, **1-8**) taken in 40 mL ethanol were added dropwise to this mixture and refluxed for 14-20 hours at 90 ºC. The reaction mixture was cooled on completion, poured onto ice cold water to get the solid arylaldoximes (**9, 11, 13, 15** and **16**) which were dried after filtration. However, arylaldoximes (**10**, **12** and **14**) were achieved by the process of extraction which was carried out with ethyl acetate and water. The organic layer of these intermediates was washed with brine, dried over anhydrous Na_2SO_4, filtered and then concentrated *in vacuo* under reduced pressure for the use in next step.

***N*-[(*E*)-Phenylmethylidene]hydroxylamine (9).** Yield: 90%; C_7H_7NO; ESI-MS (m/z): [M + H] 121.13

***N*-[(*E*)-(4-Methoxyphenyl)methylidene]hydroxylamine (10).** Yield: 85%; $C_8H_9NO_2$; ESI-MS (m/z): [M + H] 151.16

***N*-[(*E*)-(4-Methylphenyl)methylidene]hydroxylamine (11).** Yield: 82%; C_8H_9NO; ESI-MS (m/z): [M + H] 135.16

***N*-[(*E*)-(4-Ethoxyphenyl)methylidene]hydroxylamine (12).** Yield: 89%; $C_9H_{11}NO_2$, ESI-MS (m/z): [M + H] 165.18

***N*-[(*E*)-(4-Ethylphenyl)methylidene]hydroxylamine (13).** Yield: 80%; $C_9H_{11}NO$; ESI-MS (m/z): [M + H] 149.18

***N*-[(*E*)-(4-Chlorophenyl)methylidene]hydroxylamine (14).** Yield: 85%; C_7H_6ClNO; ESI-MS (m/z): [M + H] 155.58

***N*-[(*E*)-(4-Nitrophenyl)methylidene]hydroxylamine (15).** Yield: 84%; $C_7H_6N_2O_3$; ESI-MS (m/z): [M + H] 166.13

***N*-{(*E*)-[2-(Trifluoromethyl)phenyl]methylidene}hydroxylamine (16).** Yield: 91%; $C_8H_6F_3NO$; ESI-MS (m/z): [M + H] 189.13

Synthesis Protocol for Carboximidoyl Chlorides (17-24)

Each of the synthesized arylaldoxime (**9-16**, 43.91 mmol) was taken in DMF (15 mL) and stirred until dissolved. To this solution, *N*-Chlorosuccinimide (NCS) (43.91 mmol) taken in DMF (60 mL) was added dropwise and heated at 60 ºC for 8-12 hours. The progress of the reaction was monitored by using TLC. On completion, the reaction mixture was cooled at room temperature, poured onto ice cold water followed by extraction with tetrabutylmethyether (TBME). The ether layer was filtered and evaporated to dryness (at 30 ºC) to get the desired carboximidoyl chlorides **17-24** for the used in another step.

***N*-Hydroxybenzenecarboximidoyl chloride (17).** Yield: 75 %; yellow solid; C_7H_6ClNO; ESI-MS (m/z): [M + H] 155.58

***N*-Hydroxy-4-methoxybenzene-1-carboximidoyl chloride (18).** Yield: 75%; white solid; $C_8H_8ClNO_2$; ESI-MS (m/z): [M + 1] 185.60

***N*-Hydroxy-4-methylbenzene-1-carboximidoyl chloride (19).** Yield: 90%; white solid; C_8H_8ClNO; ESI-MS (m/z): [M + H] 169.60

4-Ethoxy-*N*-hydroxybenzene-1-carboximidoyl chloride (20). Yield: 90%; white solid; $C_9H_{10}ClNO_{2;}$ ESI-MS (m/z): [M + H] 199.63

4-Ethyl-*N*-hydroxybenzene-1-carboximidoyl chloride (21). Yield: 73%; yellow solid; $C_9H_{10}ClNO$; ESI-MS (m/z): [M + H] 183.63

4-Chloro-*N*-hydroxybenzene-1-carboximidoyl chloride (22). Yield: 87%; white solid; $C_7H_5Cl_2NO$; ESI-MS (m/z): [M + H] 190.02

***N*-Hydroxy-4-nitrobenzene-1-carboximidoyl chloride (23).** Yield: 86%; white solid; $C_7H_5ClN_2O_{3;}$ ESI-MS (m/z): [M + H] 200.57

***N*-Hydroxy-2-(trifluoromethyl)benzene-1-carboximidoyl chloride (24).** Yield: 93%; white solid; $C_8H_5ClF_3NO$; ESI-MS (m/z): [M + H] 223.5

X-ray crystallography data

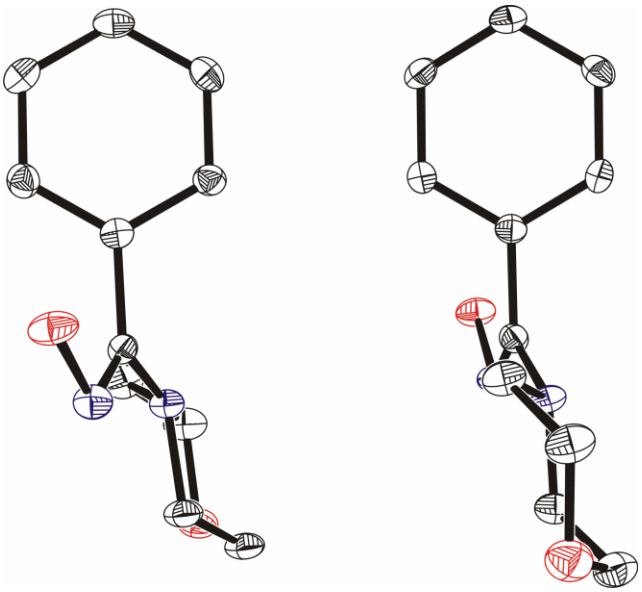


Figure S1. Atropoisomers present in **25**.

Table S1. Bond lengths [Å] and angles [°] for the compounds **25, 28** and **32**

Bond lengths	**25**	**28**	**32**
O(1)-N(1)	1.431(2)	1.4276(13)	1.4225(16)
N(1)-C(1)	1.289(3)	1.2904(15)	1.285(2)
C(1)-N(2)	1.389(3)	1.3951(15)	1.387(2)
N(2)-C(9)			
N(2)-C(10)			
O(2)-N(4)			
O(3)-N(4)			
O(2)-C(9)	1.433(5)	1.4280(16)	1.428(2)
O(2)-C(10)	1.428(4)	1.4309(15)	1.432(2)
N(2)-C(8)	1.478(3)	1.4652(15)	1.471(2)
N(2)-C(11)	1.459(3)	1.4731(15)	1.475(2)
Angles	**25**	**28**	**32**
C(1)-N(1)-O(1)	111.7(2)	112.69(10)	113.74(12)
N(1)-C(1)-N(2)	117.8(2)	117.31(10)	126.21(14)
C(9)-N(2)-C(10)			
C(9)-N(2)-C(1)			
C(10)-N(2)-C(1)			
C(10)-N(3)-C(8)			
C(1)-N(2)-C(11)	117.63(19)	117.40(10)	118.55(13)
C(1)-N(2)-C(8)	114.9(2)	116.68(9)	118.51(12)
C(11)-N(2)-C(8)	111.2(2)	111.24(10)	110.87(12)
C(9)-O(2)-C(10)	108.4(3)	109.87(10)	109.72(12)

Table S2. Hydrogen bonds in the compounds **25, 28** and **32**.

D-H...A	**compound**	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O(1)-H(1O)...N(3)#1	**25**	0.90(3)	1.93(3)	2.796(3)	163(3)
O(1)-H(1O)...O(3)#1	**25**	0.90(3)	2.57(3)	3.2211(19)	130(3)
O(3)-H(3O)...N(1)#2	**25**	0.94(4)	1.91(4)	2.810(3)	159(3)
O(3)-H(3O)...O(1)#2	**25**	0.94(4)	2.57(4)	3.2211(19)	127(3)
O(1)-H(1O)...N(1)#3	**28**	0.89(2)	1.93(2)	2.7838(14)	160.8(18)
O(1)-H(1O)...O(1)#3	**28**	0.89(2)	2.59(2)	3.2118(18)	127.9(15)
O(1)-H(1O)...N(1)#4	**32**	0.96(3)	1.83(3)	2.7398(18)	158(2)
O(1)-H(1O)...O(1)#4	**32**	0.96(3)	2.57(2)	3.226(2)	125.5(18)

Symmetry transformations used to generate equivalent atoms:

#1 x,y-1,z #2 x,y+1,z #3 -x-2,-y,-z+1 #4 -x,-y+1,-z

Molecular docking

Table S3. Molecular docking results showing the binding energy and specific interacting residues of MARK4 with each synthesized target chemotypes **25-32**

Compound	Docking score (Kcal/mol)	Protein-ligand interactions		
		Hydrogen bonds		Other interacting residues
		Amino acid residues	Distance (Å)	
25	-6.2	Lys85	3.1	Val70, Ala83, Lys85, Val116, Mat132, Glu133, Asn183, Leu185, Ala195, Asp196
26	-6.5	Gly65 Glu182	3.3 3.2	Gly63, Lys64, Gly65, Ala68, Lys69, Val70, Ala83, Lys85, Val116, Met132, Glu182, Asn183, Ala195, Asp196
27	-6.8	Asn183	2.1	Ile62, Gly63, Val70, Ala83, Lys85, Val116, Met132, Glu182, Asn183, Leu185, Ala195, Asp196
28	-6.8	Gly65 Asn183	3.3 3.2	Gly63, Lys64, Gly65, Ala68, Lys69, Val70, Ala83, Ile84, Lys85, Val116, Met132, Glu182, Asn183, Ala195, Asp196
29	-7.1	Gly65 Glu182	3.2 2.6	Gly63, Lys64, Gly65, Ala68, Lys69, Val70, Ala83, Lys85, Val116, Met132, Glu182, Asn183, Ala195, Asp196
30	-6.6	None	None	Ile62, Gly63, Val70, Ala83, Lys85, Val116, Met132, Glu182, Asn183, Leu185, Ala195, Asp196
31	-7.0	Lys85	2.8	Gly63, Val70, Ala83, Ile84, Lys85, Val116, Leu130, Met132, Glu133, Glu139, Glu182, Asn183, Leu185, Ala195, Asp196
32	-6.9	Gly65 Glu182	3.1 2.5	Gly63, Lys64, Gly65, Ala68, Lys69, Val70, Lys85, Met132, Glu182, Asn183, Leu185, Ala195, Asp196

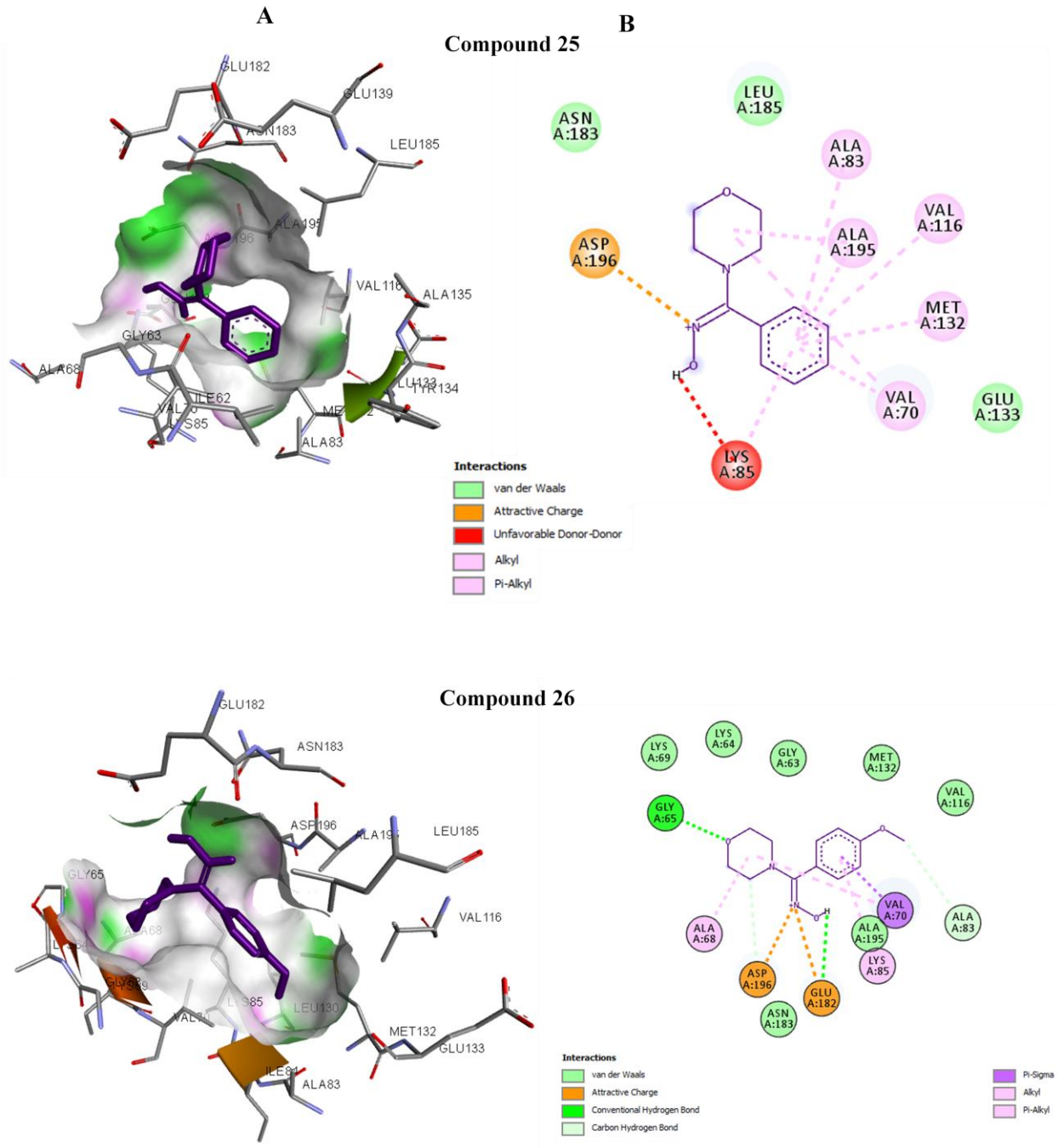


Figure S2. Molecular docking studies of synthesized compounds with MARK4: View of the catalytic pocket of MARK4 with **(A)** compound **25** and compound **26 (B)** 2D schematic representation of the docking models of compound **25** and compound **26**. Dotted lines in different colours reflected various types of interaction such as hydrogen bonding, charge or polar interactions, van der Waals and π-sigma interactions

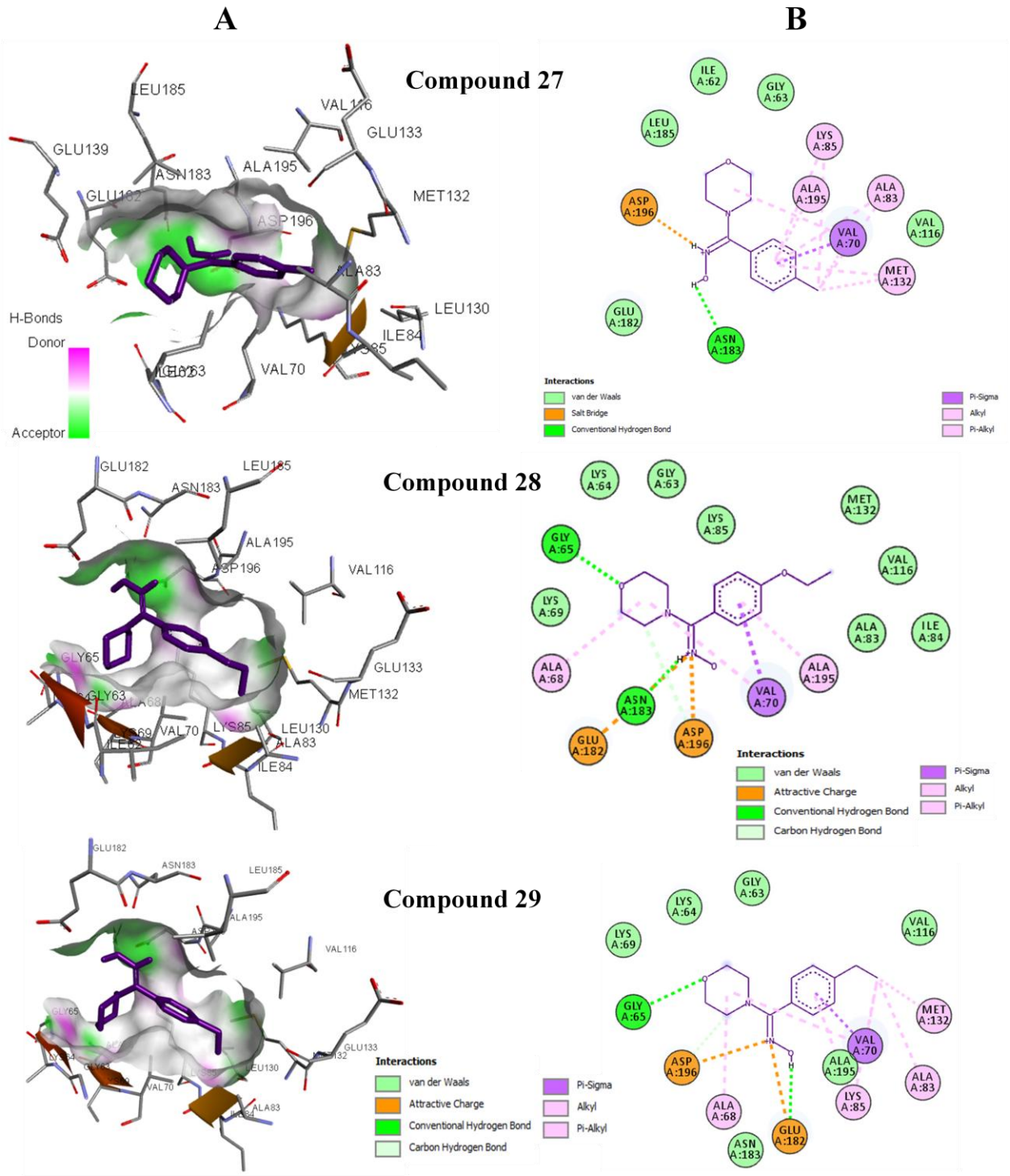


Figure S3. Molecular docking studies of synthesized compounds with MARK4: View of the catalytic pocket of MARK4 with **(A)** compound **27**, compound **28** and compound **29 (B)** 2D schematic representation of the docking models of compound **27,** compound **28** and compound **29**. Dotted lines in different colours reflected various types of interaction such as hydrogen bonding, charge or polar interactions, van der Waals and π-sigma interactions

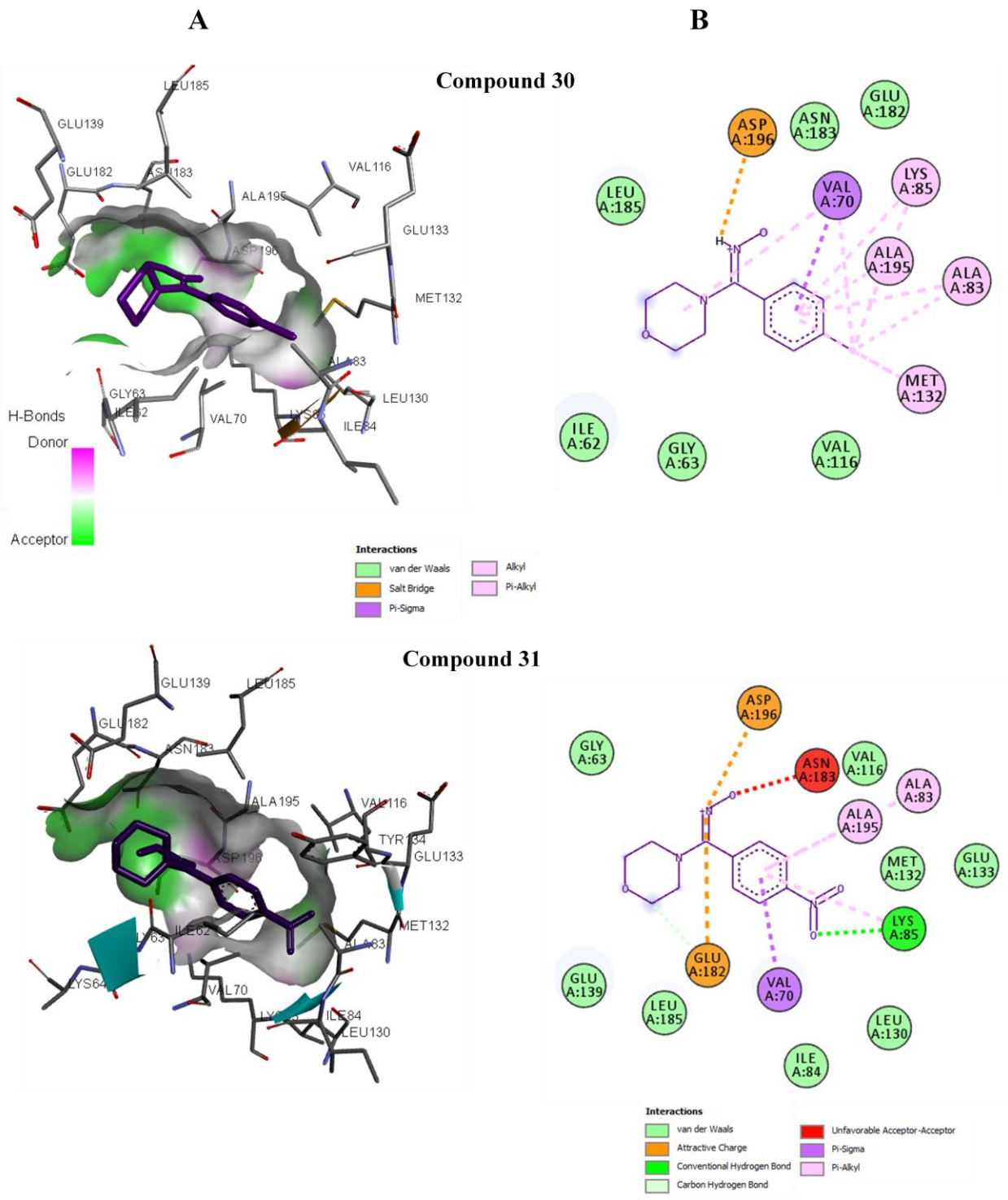


Figure S4. Molecular docking studies of synthesized compounds with MARK4: View of the catalytic pocket of MARK4 with **(A)** compound **30** and compound **31 (B)** 2D schematic representation of the docking models of compound **30** and compound **31**. Dotted lines in different colours reflected various types of interaction such as hydrogen bonding, charge or polar interactions, van der Waals and π-sigma interactions

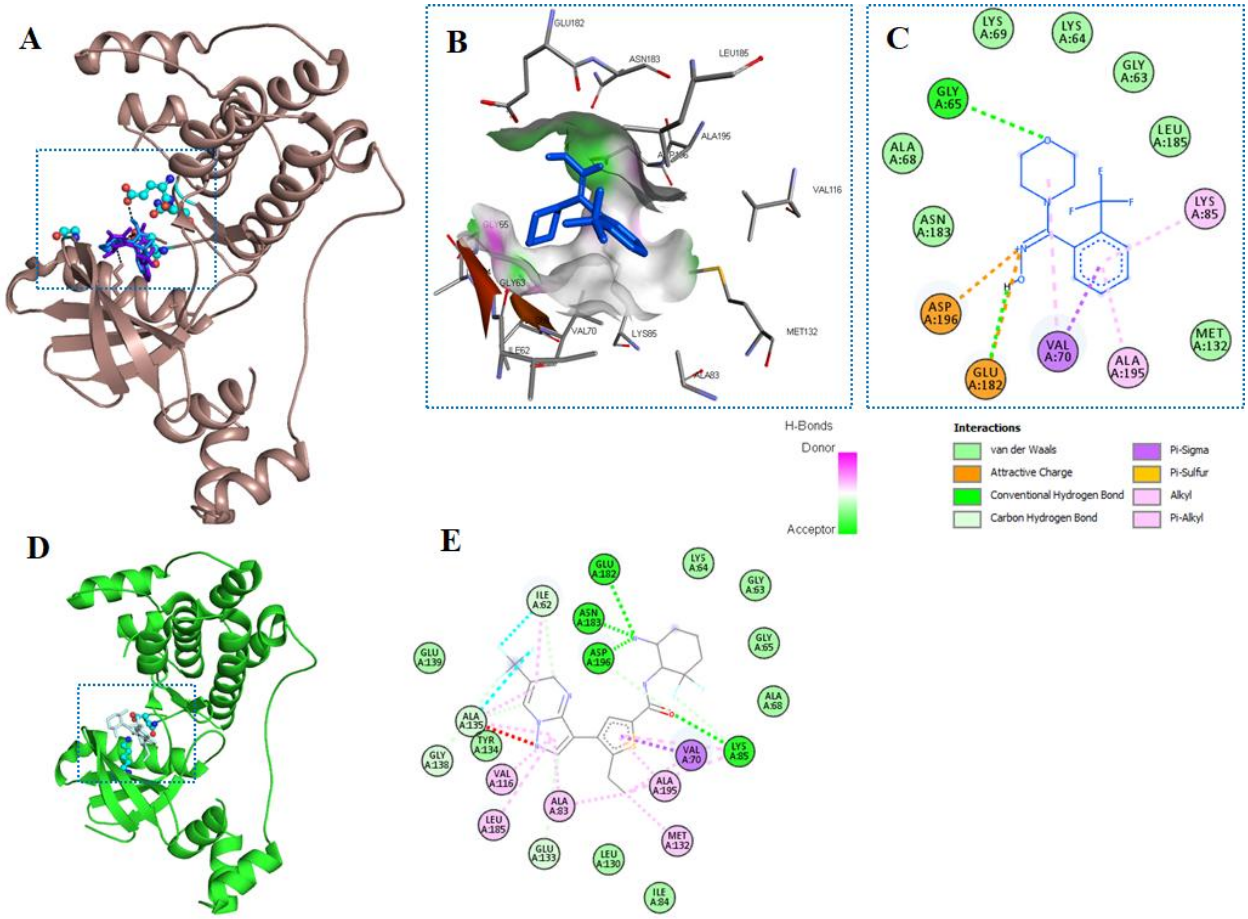


Figure S5. (A) 3D presentation of MARK4 docked ligand complex of compound **32** (blue) to the active site residues of MARK4. **(B)** Focused view of MARK4 binding pocket with compound **32** shows the hydrogen bond donor-acceptor residues of protein. **(C)** 2D representation of residues involved in different interactions like van der Waals interactions, hydrogen bonding, charge or polar interactions (each type of interaction is represented by respective color, see inset). **(D)** 3D presentation of MARK4 re-docked with reported co-crystal ligand pyrazolopyrimidine inhibitor (light blue), PDB ID: 5ES1. **(E)** 2D representation of MARK4 residues involved in different interactions with reported co-crystal ligand pyrazolopyrimidine inhibitor.

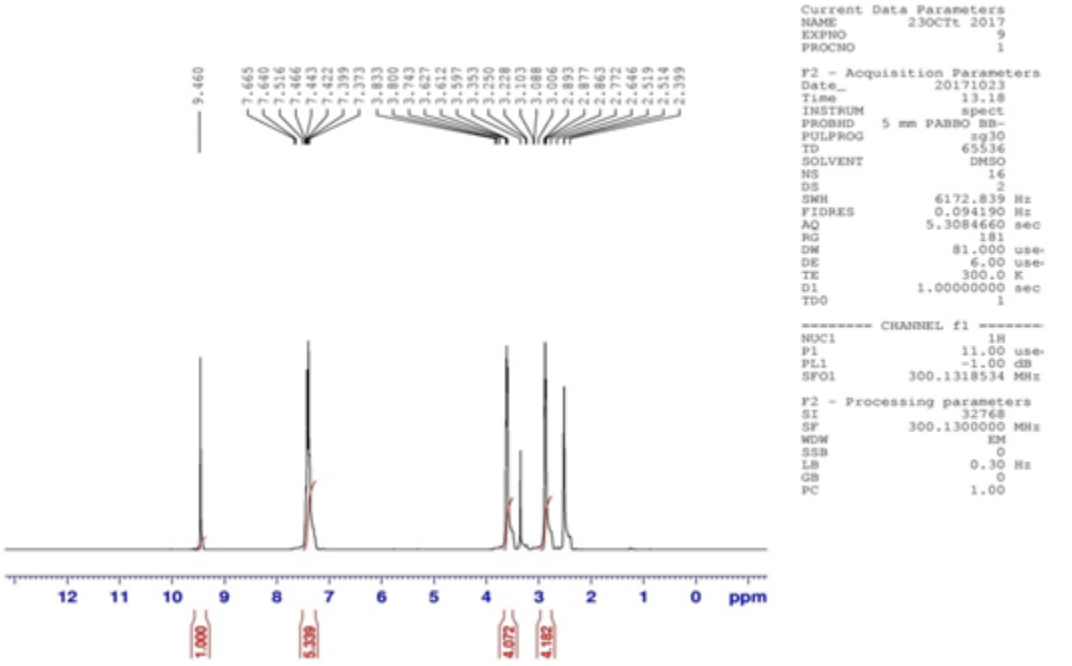
Single Dose Kinase Inhibition Profiling of Compound 32

Table S4. Kinase selectivity profiling of compound **32** with 30 kinases of Ser/Thr family using kinase screening kit (Promega, Madison, USA) and malachite green assay.

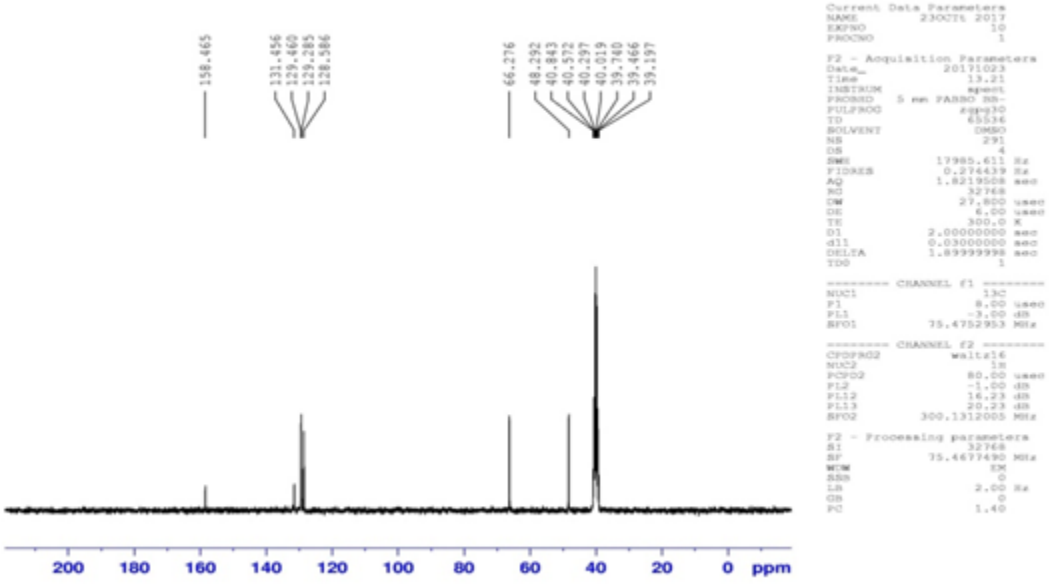
S.NO.	Kinase	% inhibition (at 10 µM) of compound 32
1.	Positive control (MARK4)	**96.93**
2.	Negative control	0.38
3.	MAPKAPK2	41.22
4.	CHK1	1.50
5.	CHK2	3.49
6.	MARK1	2.12
7.	MELK	10.93
8.	PASK	17.72
9.	PIM1	10.27
10.	CAMK2 alpha	19.12
11.	CAMK2beta	15.45
12.	CAMK4	23.56
13.	CAMKI	12.11
14.	CAMKII	6.55
15.	CHKtide	9.85
16.	ZIPtide2	13.70
17.	ZIPtide	1.72
18.	AMPKA1	3.26
19.	AMPKB1	16.56
20.	MBP	14.64
21.	SAMStide	9.42
22.	S6K	15.05
23.	PKCu	10.22
24.	CREBtide	8.46
25.	MBP2	12.32
26.	HSP27tide	24.72
27.	STK33	8.42
28.	DAPK1	23.52
29.	ILK	9.24
30.	PDK3	23.42
31.	FASTK	12.11

Table S5. Solubility of the compounds **25-32** (mg/mL)

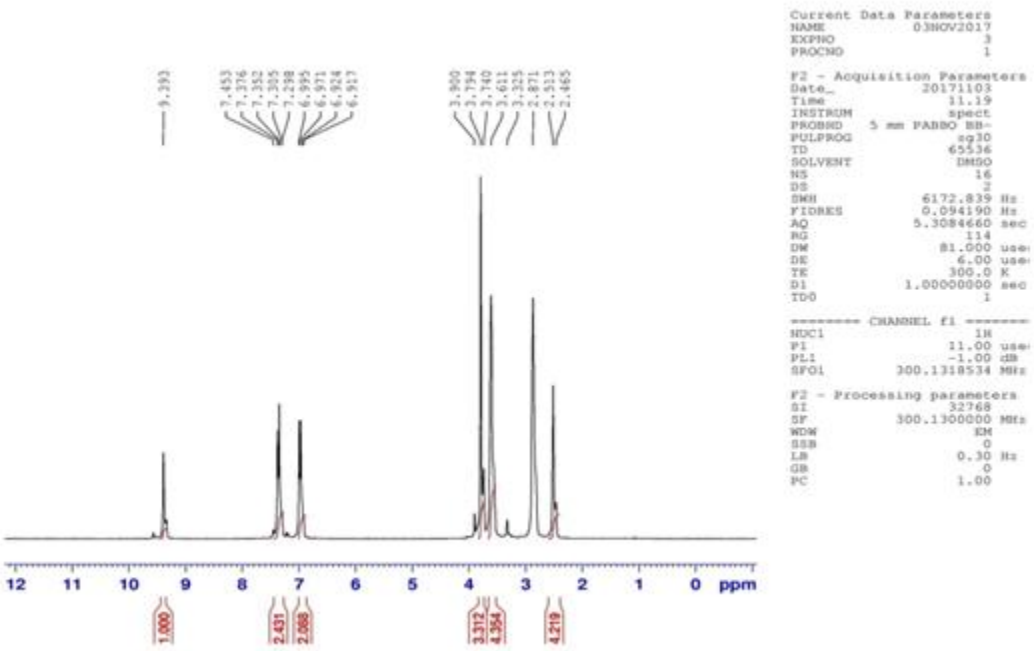
Compound No.	**Aqueous Solubility (S) mg/mL**
25	24±0.01
26	30±0.4
27	48±0.7
28	32±0.7
29	46±0.8
30	54±0.9
31	76±1.5
32	85±1.1



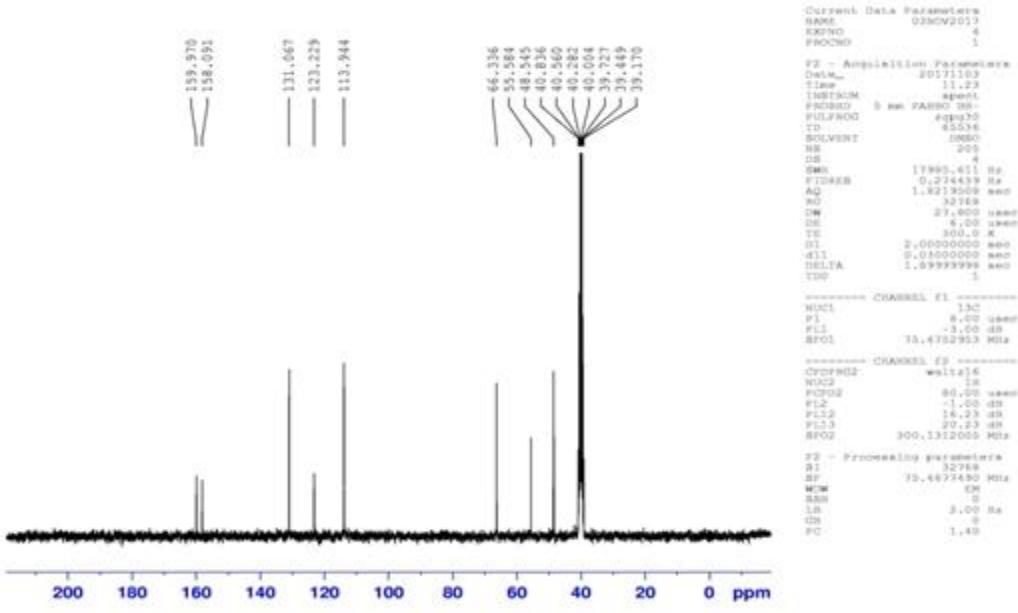
[1]H NMR of Compound 25



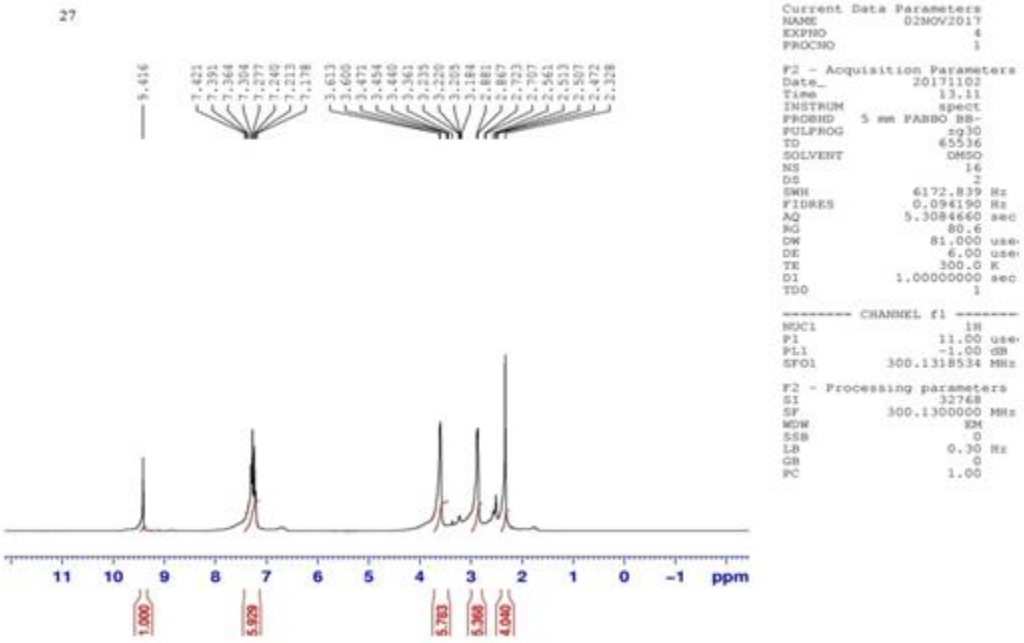
[13]C NMR of Compound 25



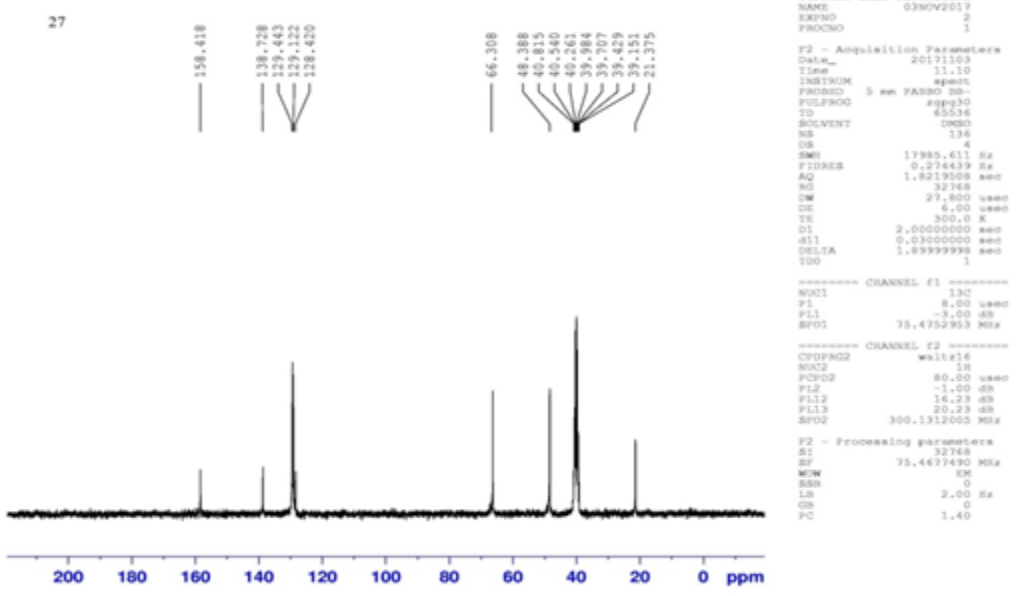
[1]H NMR of Compound 26



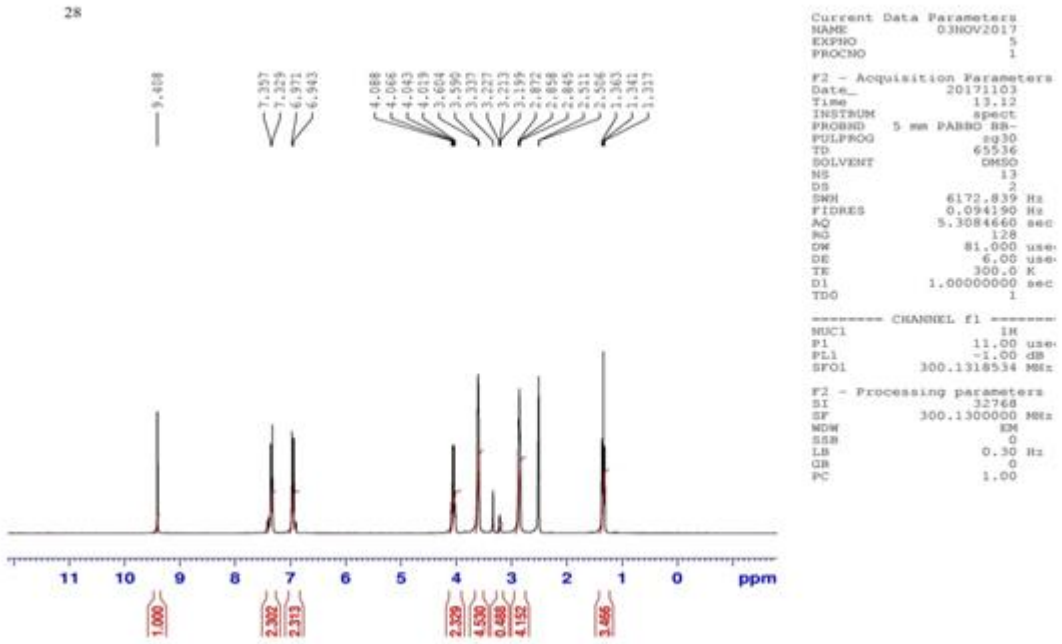
[13]C NMR of Compound 26



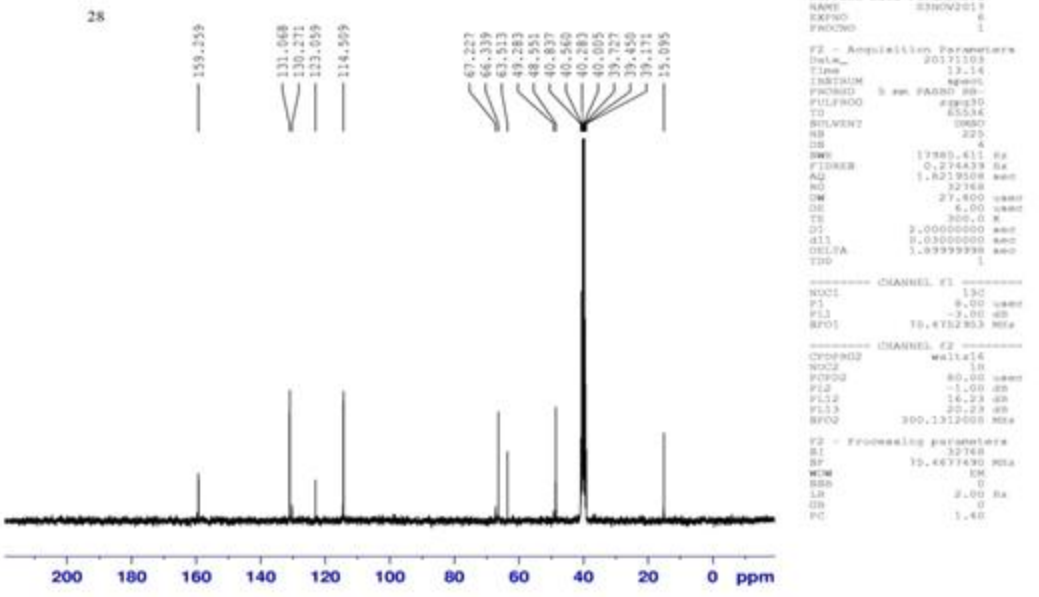
[1]H NMR of Compound 27



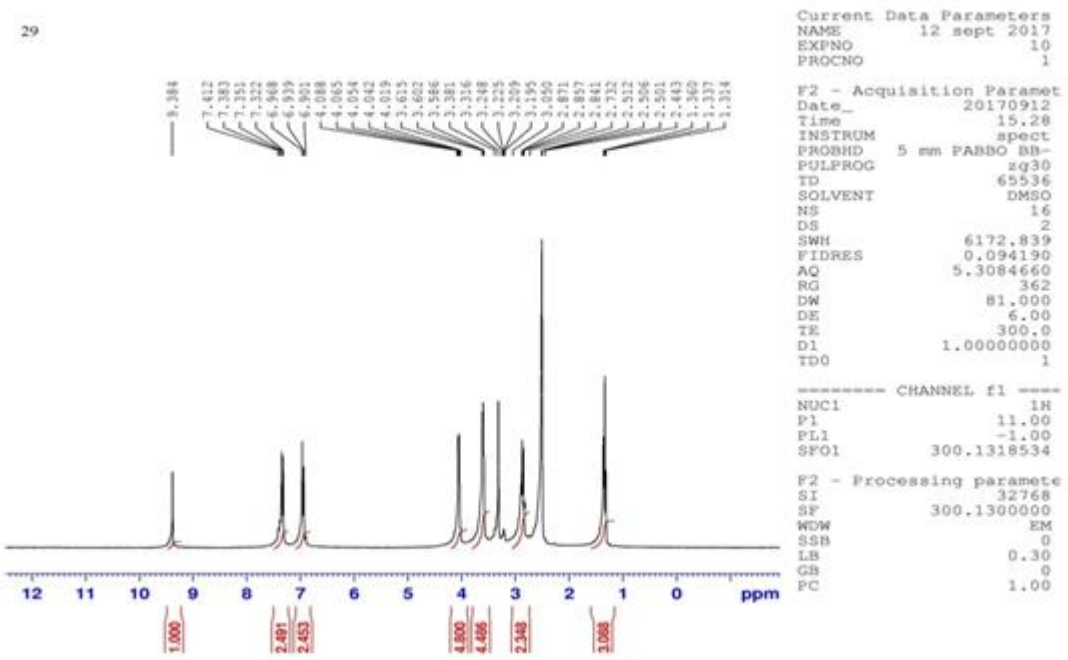
[13]C NMR of Compound 27



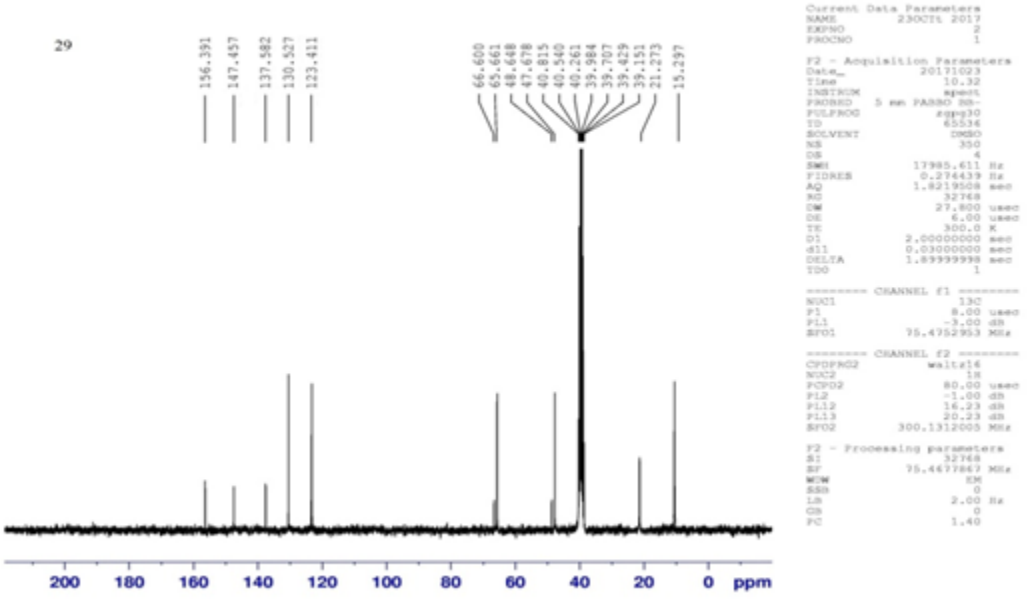
[1]H NMR of Compound 28



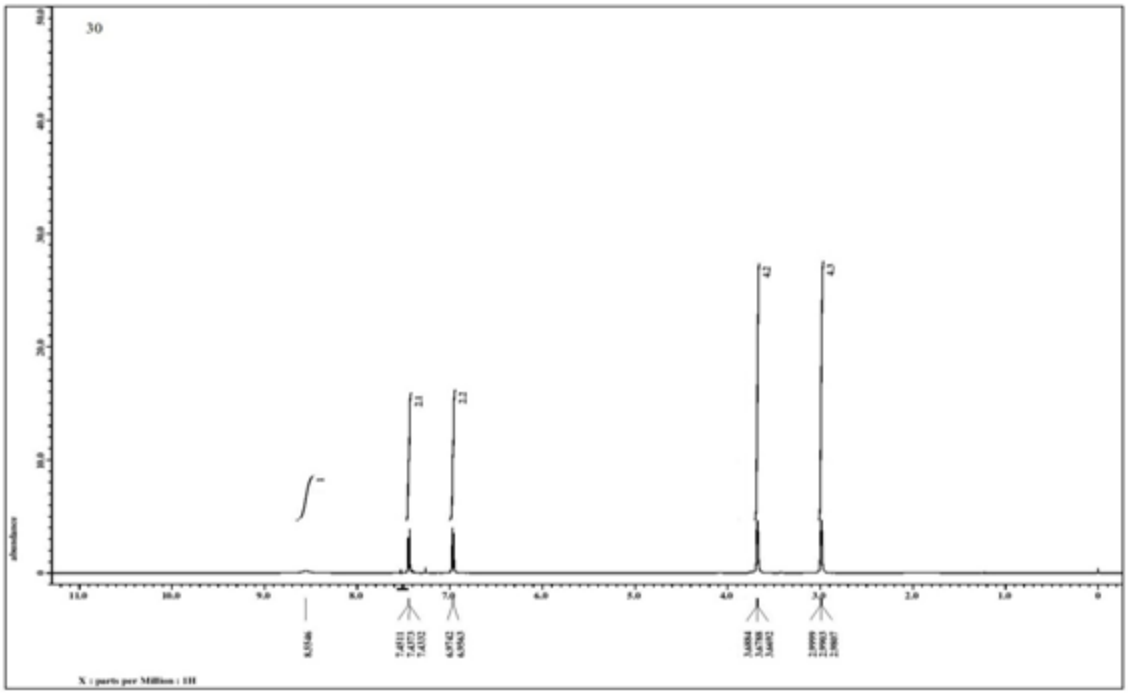
[13]C NMR of Compound 28



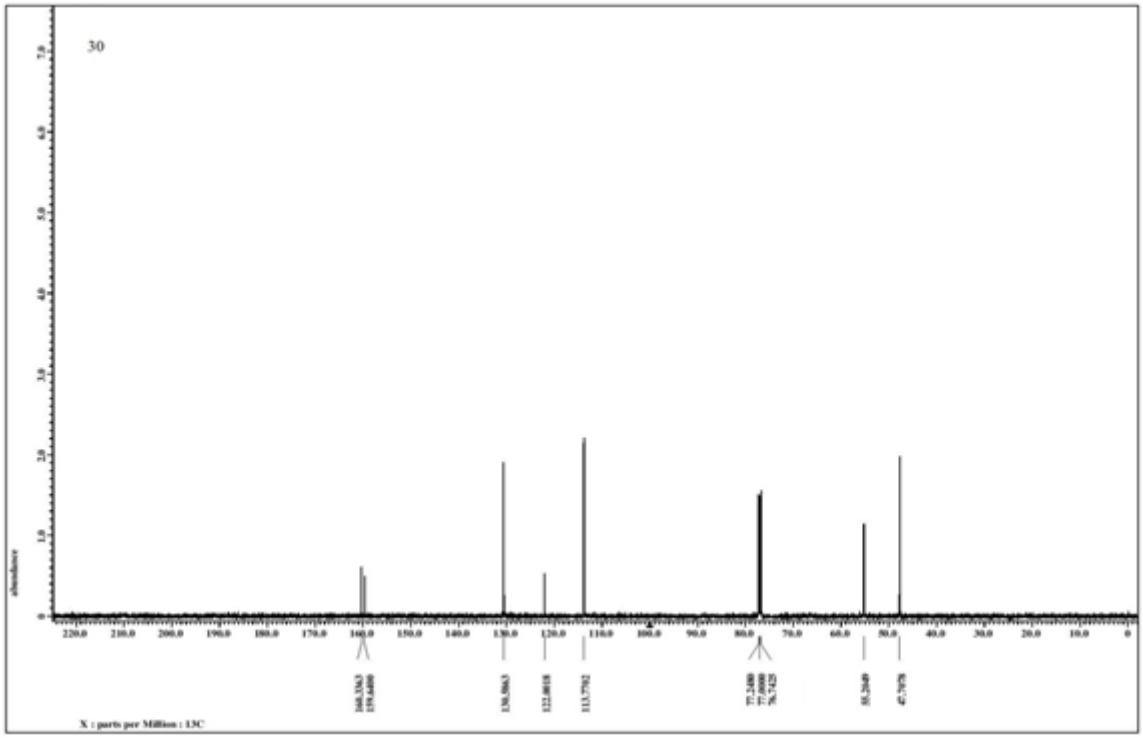
[1]H NMR of Compound 29



[13]C NMR of Compound 29



[1]H NMR of Compound 30



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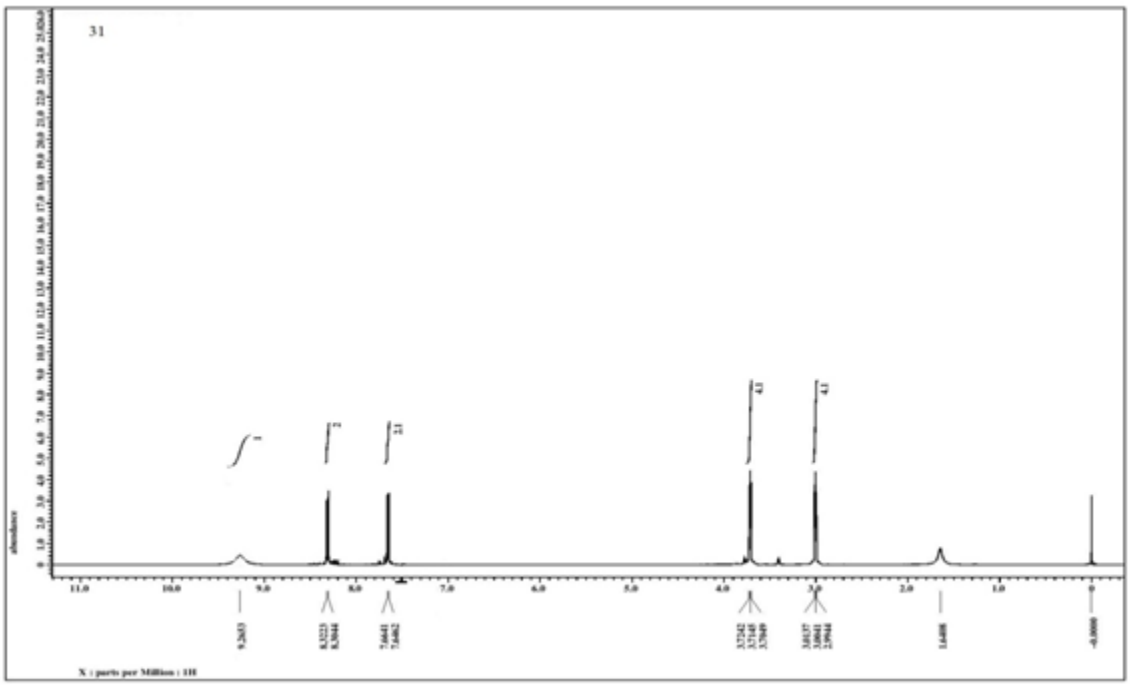

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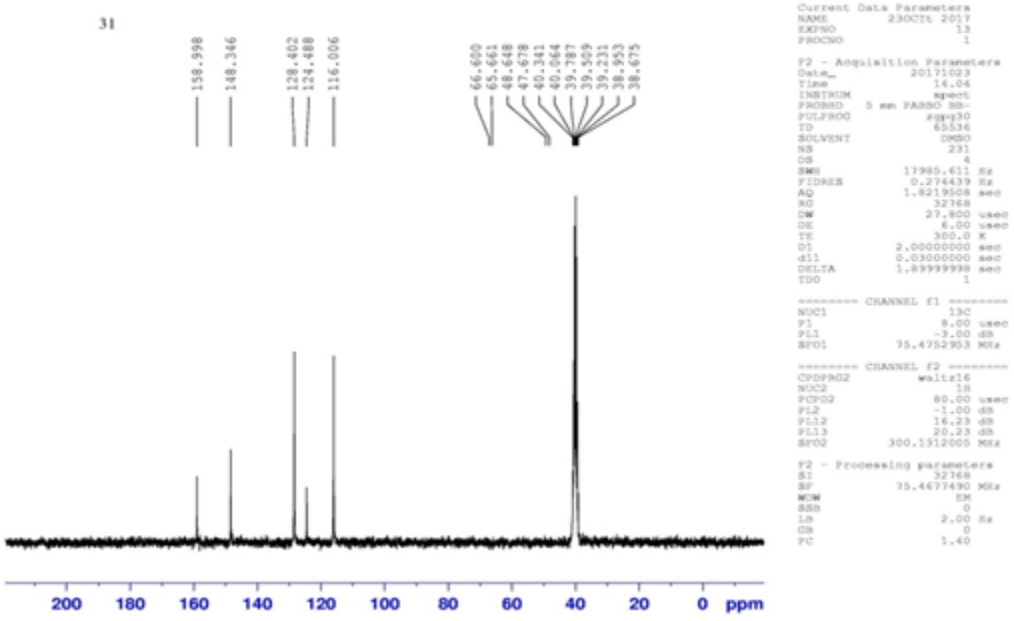

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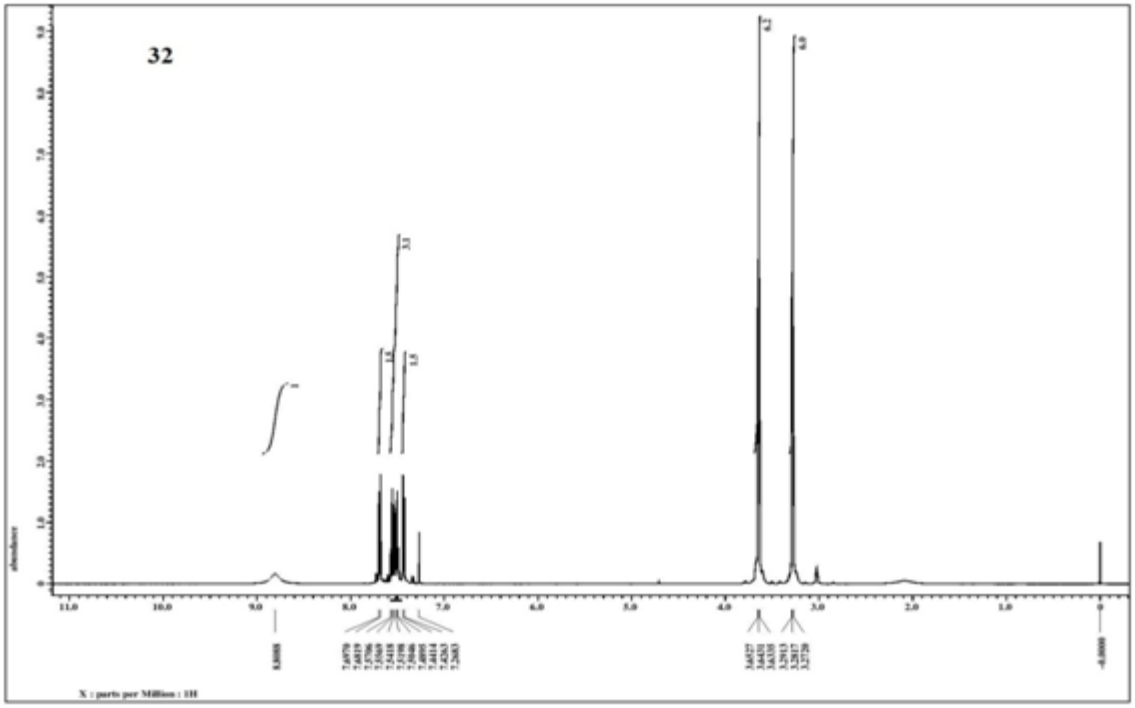

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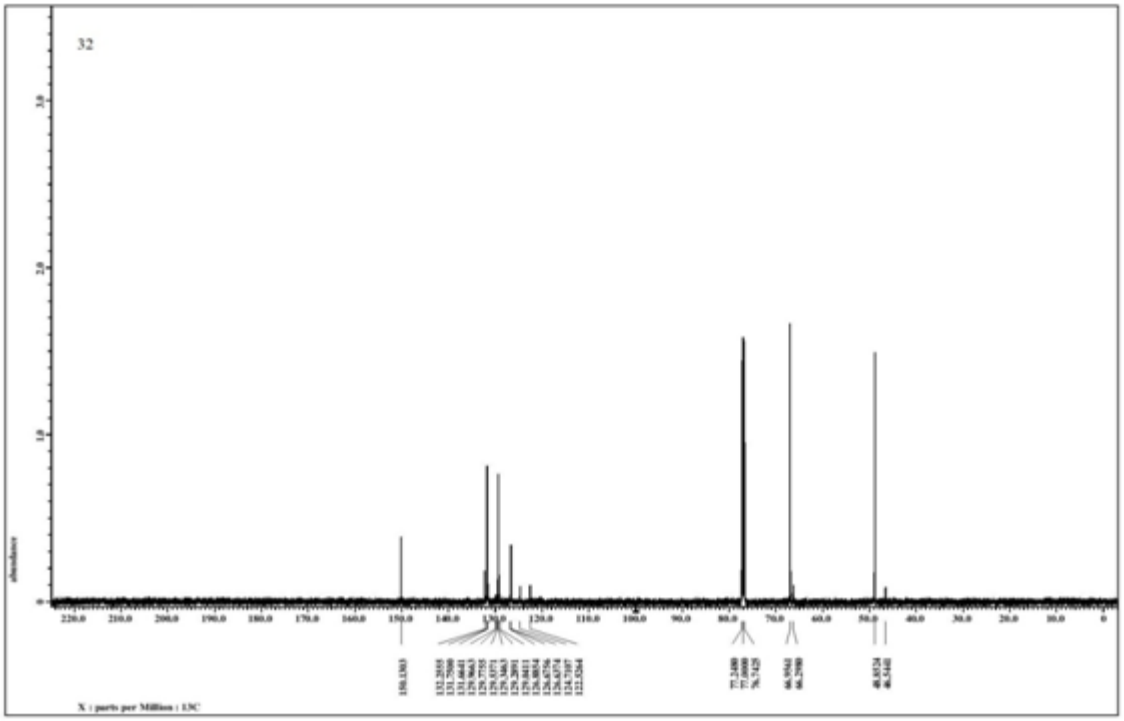

[13]C NMR of Compound 32