Design and Synthesis of Nucleic Acid Nano-environment Interactome-Targeting Small Molecule PROTACs and Their Anticancer Activity

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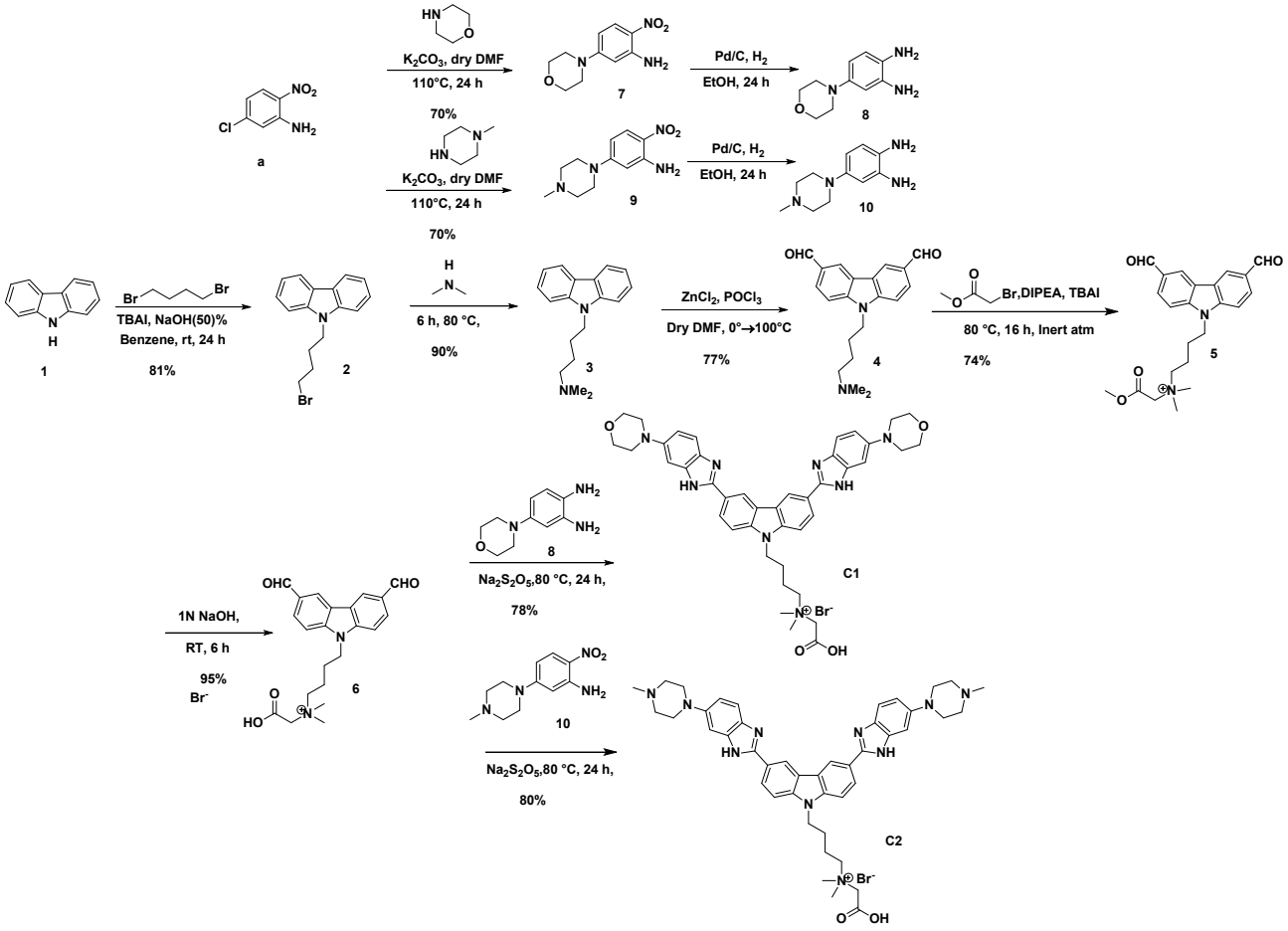
Experimental protocol

Materials and Methods

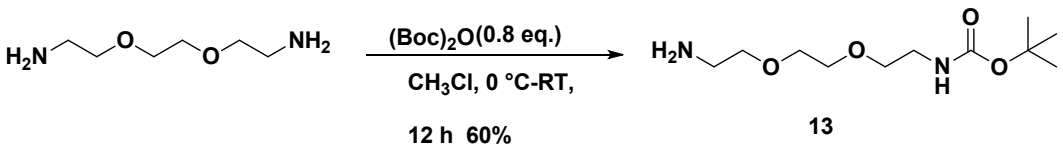
All the chemicals and solvents in analytical grades were used directly. All chemicals were purchased from Sigma-Aldrich, Spectrochem, SRL or otherwise stated and used as received. ^{1}H NMR experiments were performed using Bruker Avance 500 and 700 MHz spectrometers at 298 K. ESI-MS acquisition was carried out XEVO-G2-XS-QTOF spectrometer (Waters).

Complete Synthetic Scheme

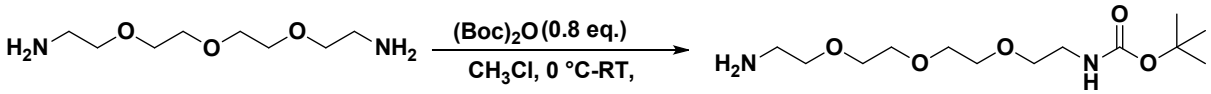
A. Synthesis of G4 binding Ligand



B. Synthesis of PEG Linker amine

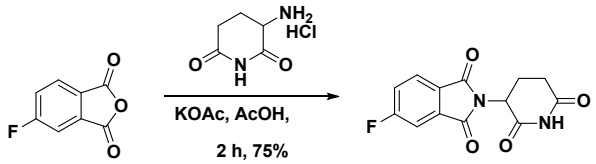


13



14

C. Synthesis of (E3 -ligase) CRBN binding Ligand



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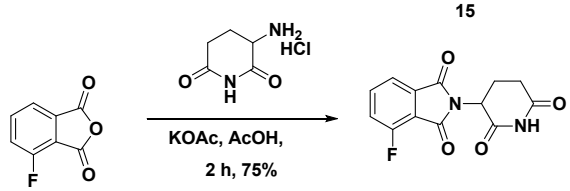
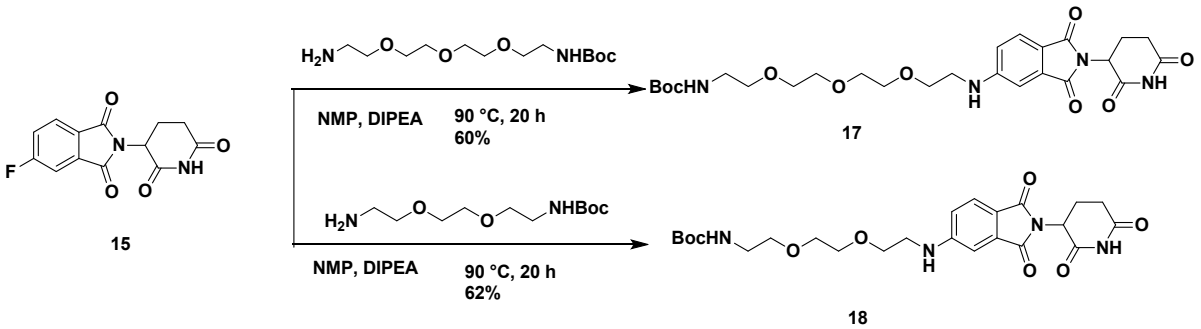


16

D. Conjugation of the linker and fluorothalidomide conjugates.



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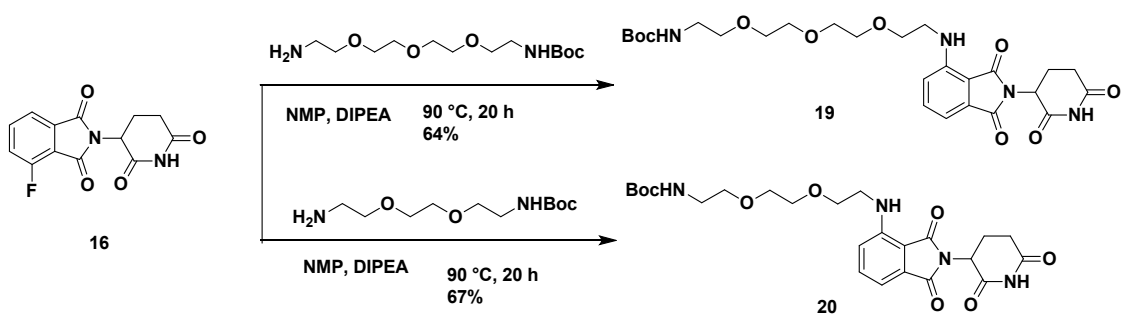
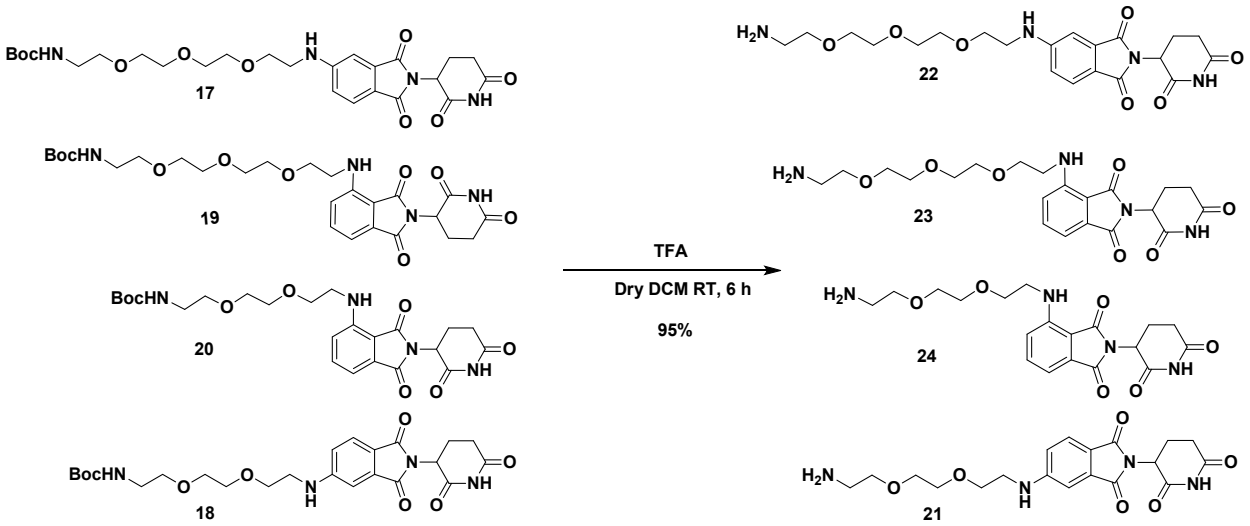


16

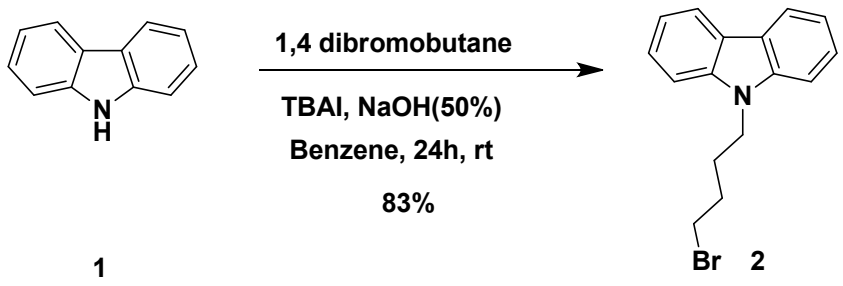
19

20

E. Deprotection of Boc-protected thalidomide linker



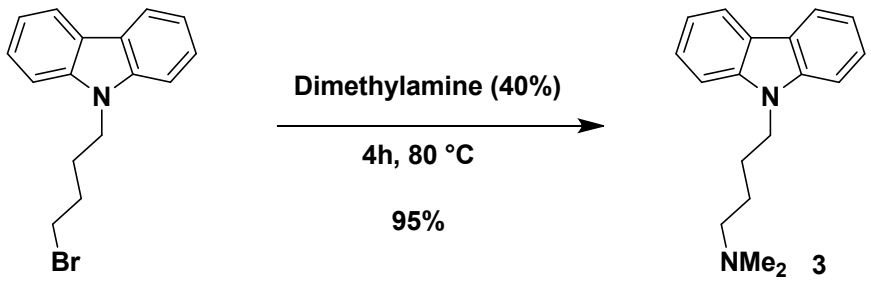
9-(4-bromobutyl)-9H-carbazole (2)



A mixture of TBAI (353.6mg, 369.41 mmoles), Carbazole **(1)** (4g, 23.92 mmoles), and 1,4dibromobutane (41.28mg, 191.36 mmoles), in benzene (30ml) and aqueous NaOH (50%,18ml) was taken in a 250ml round bottom flask at the room temperature. This mixture was then stirred for 24h at room temperature. The reaction was monitored on the TLC. After

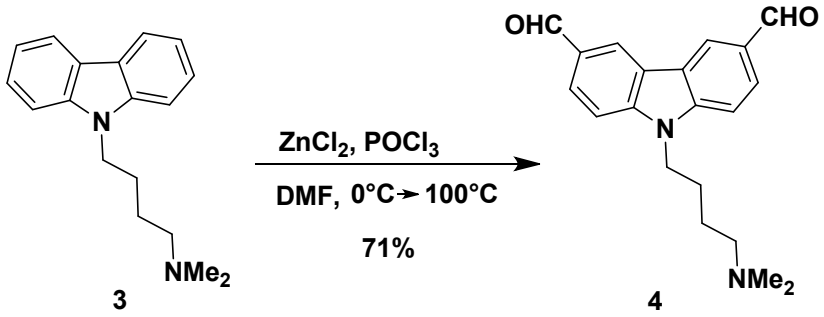
the completion of the reaction, the organic layer was separated from the reaction mixture by extracting with ethyl acetate and passing it through a bed of dry Na_2SO_4 and evaporated to get an impurified product which was purified by silica gel column chromatography with hexane/ethyl acetate mixture as the eluent. The pure product was collected at 8% ethyl acetate/hexane, which was confirmed by the TLC. The product was further purified and concentrated by evaporating all the solvents, resulting in a white crystalline solid (83%) **(2)**

4-(9H-carbazol-9-yl)-N,N-dimethylbutan-1-amine (3)

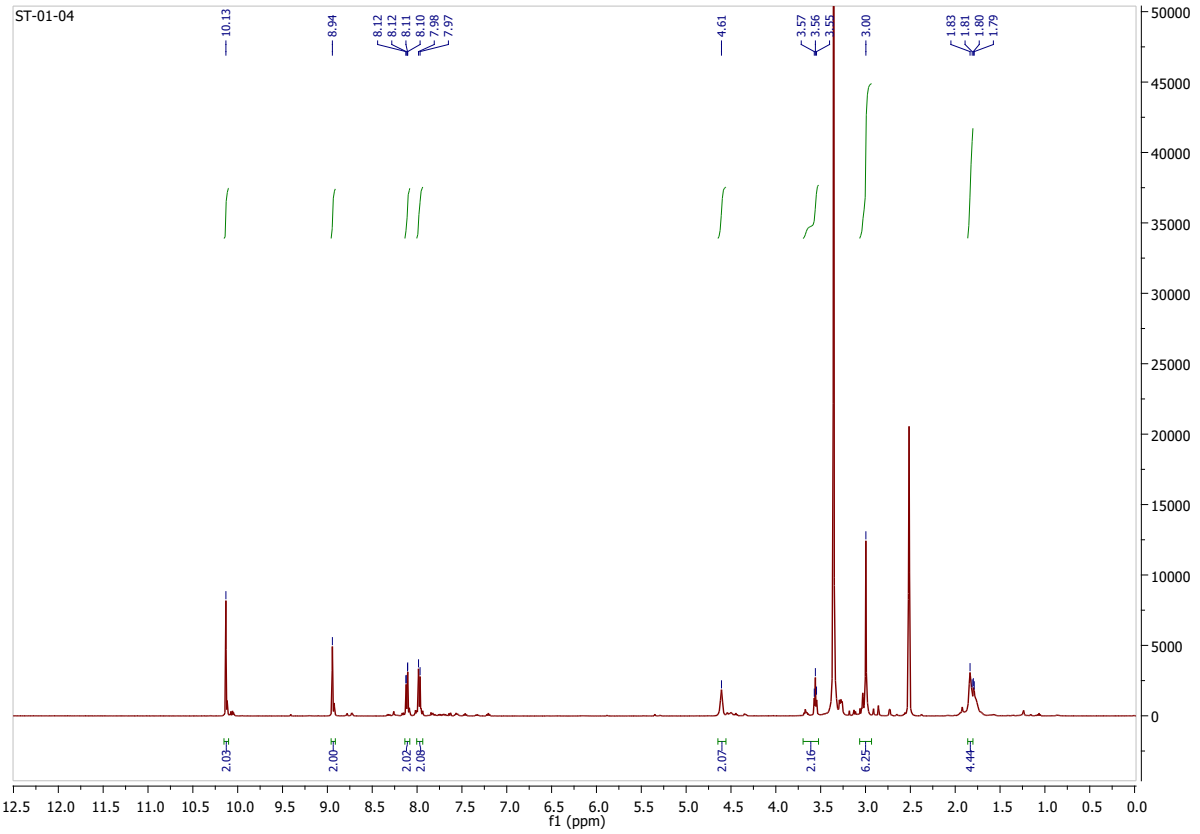


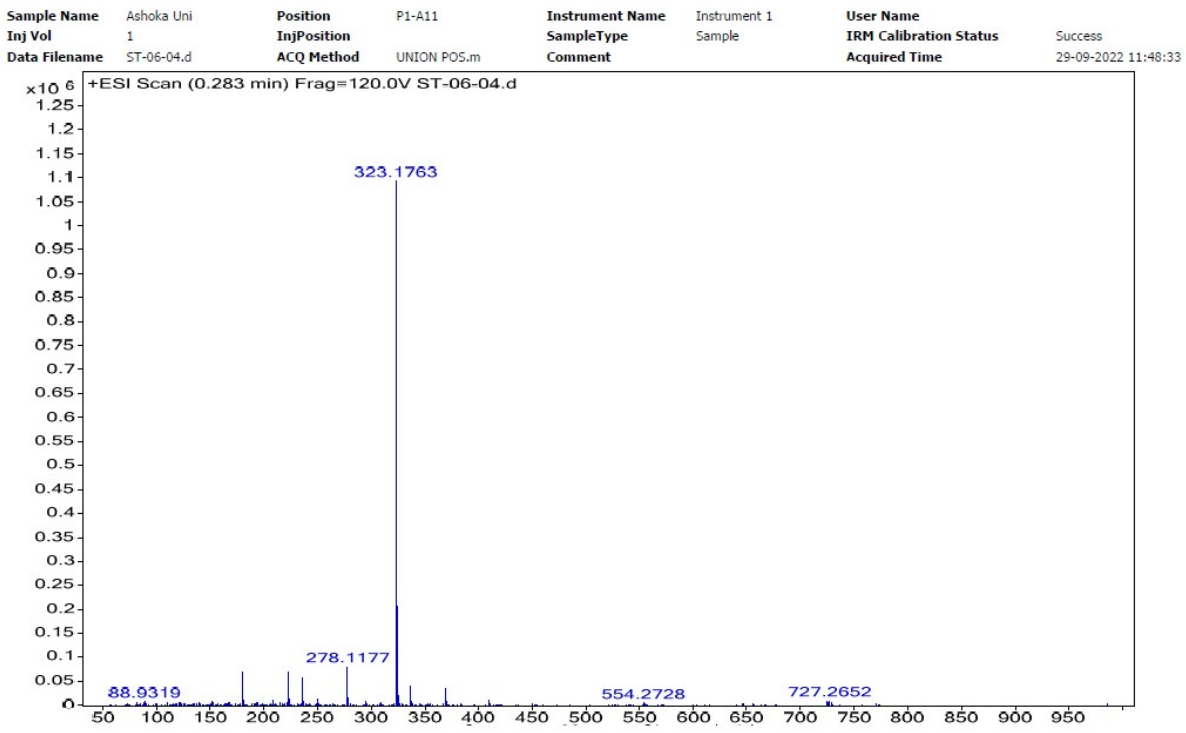
The N-bromobutylcarbazole **(2)** (5.4g) was dissolved in 20ml of acetonitrile and the dimethylamine gas (40%) was passed into it for 6h at 80 ^{0}C. The reaction progress was monitored on TLC, and after the completion of the reaction, it was again run for an hour at room temperature, to get rid of excess dimethylamine gas. The reaction mixture was purified by evaporating all the solvent under high vapor pressure. The product was purified by silica gel column chromatography with a chloroform/methanol mixture as the eluent. The pure product (3) came at 10% MeOH/$CHCl_3$ as an off-white gummy viscous solid (4g, 95%) which was confirmed to be pure by TLC.

9-(4-(dimethylamino)butyl)-9H-carbazole-3,6-dicarbaldehyde (4)

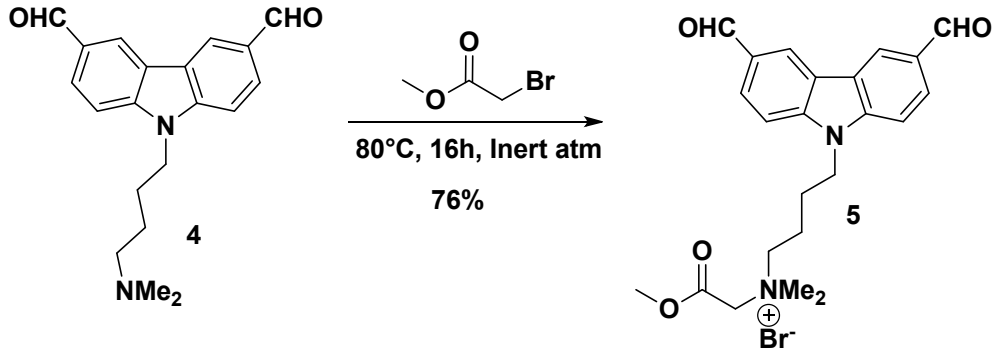


The mixture of Compound **3** (1.5 g, 5.6 mmol) and 1.50 mg (6.24 mmol) of anhydrous $ZnCl_2$ (activated) was taken in a oven dried 2-neck round-bottom flask (100mL) with 12 mL of dry DMF. This mixture was heated for 30 min at 100 ^{0}C and was then cooled to room temperature. To this reaction mixture, 5.6 mL (31.2 mmoles) of $POCl_3$ was added gradually and dropwise, upon constant cooling over an ice bath for 25 min followed by again heating at 100 °C for 24 h. Then, the reaction mixture was concentrated by removing the excess DMF under high vapor pressure and quenched cautiously with the addition of 10 ml of ice-cold water. Then the reaction, the mixture was neutralized with a concentrated KOH solution, over an ice bath, basic pH was confirmed by pH paper. The mixture was finally extracted with ethyl acetate and washed with water. The organic layer was isolated by passing through a bed of dry Na_2SO_4. The filtrate was collected and concentrated to get a reddish-brown gummy mass which was purified by silica gel column chromatography using chloroform/methanol as the eluent. The final product came at 17% MeOH/$CHCl_3$ as a maroon-brown powdered solid **(4)** which was confirmed to be pure by TLC (10% MeOH/$CHCl_3$) (1.33g,73.5%).**^{1}H NMR (400 MHz, $CDCl_3$)**: δ 10.15 (s, 2H), 8.62 (s, 2H), 8.09 (d, J = 8.0Hz, 2H), 7.58 (d, J = 8.0 Hz, 2H), 4.43 (t, J = 8 Hz, 2H), 2.38-2.44 (s,6H), 1.27(s, 6H). **ESI-HRMS (m/z)** [M+H$^+$] Cald. = 323.1761, found = 323.1763

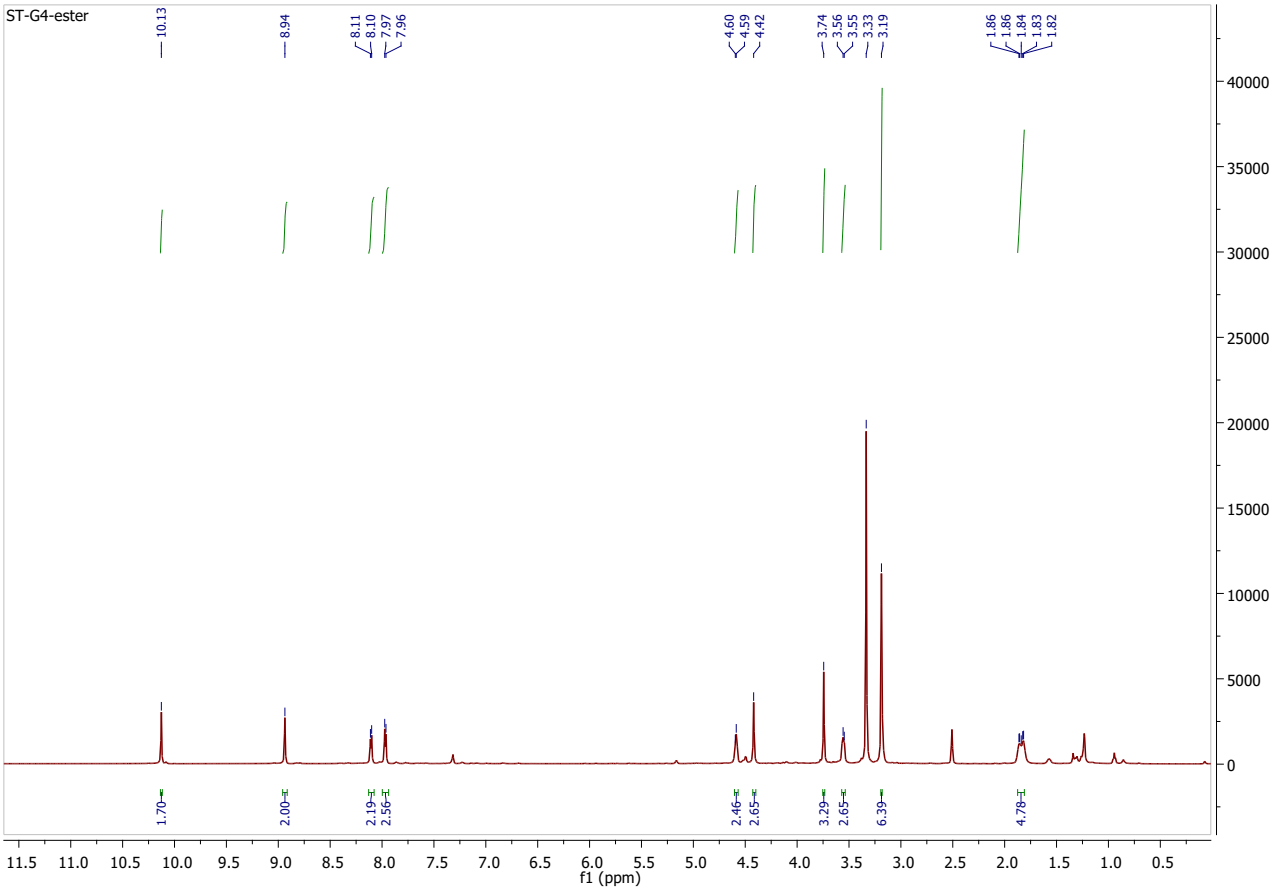




4-(3,6-diformyl-9H-carbazol-9-yl)-N-(2-methoxy-2-oxoethyl)-N,N-dimethylbutan-1-aminium (5)



To the compound **4** (90mg, 0.27mmoles) in dry acetonitrile TBAI (42mg, 0.324mmoles), DIPEA (206mg, 1.35mmoles), methyl bromoacetate (206mg, 1.35mmoles), was added and stirred at 80 for 16h. The reaction mixture was extracted with chloroform and a minimum amount of water, purified by silica gel Column Chromatography, the product was characterized to be pure by **^{1} H NMR (700MHz, CD_3OD)**: δ 10.13 (s, 2H), 8.94 (s, 2H), 8.10 (d, j = 14Hz, 2H), δ7.96 (d, j = 14Hz, 2H), 4.59 (m,2H), 4.42 (s, 2H), 3.74 (s, 3H), 3.19 (s, 6H), 1.86-1.82 (m,4H). **ESI-HRMS (m/z)** [M^+] Cald. =395.1965, found = 395.2016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3.19
1.86
1.86
1.84
1.83
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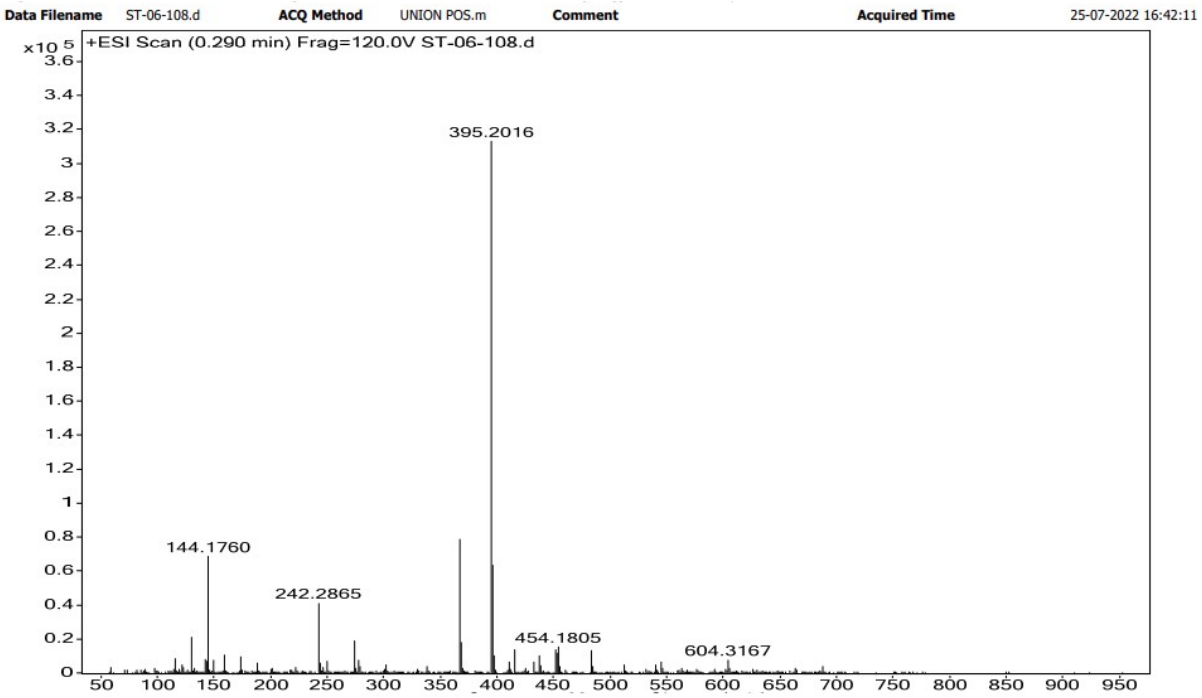
f1 (ppm)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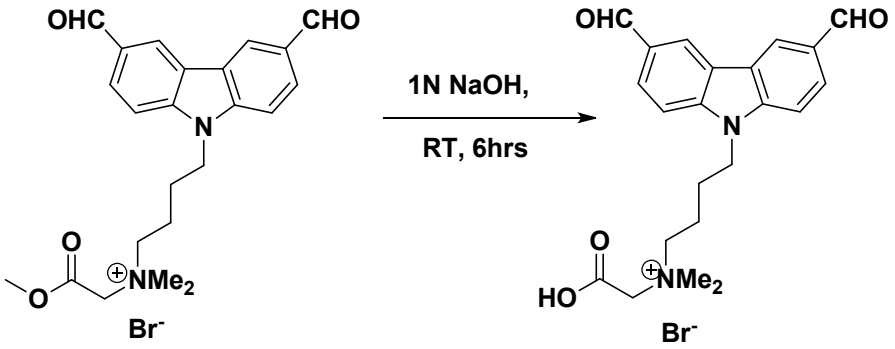
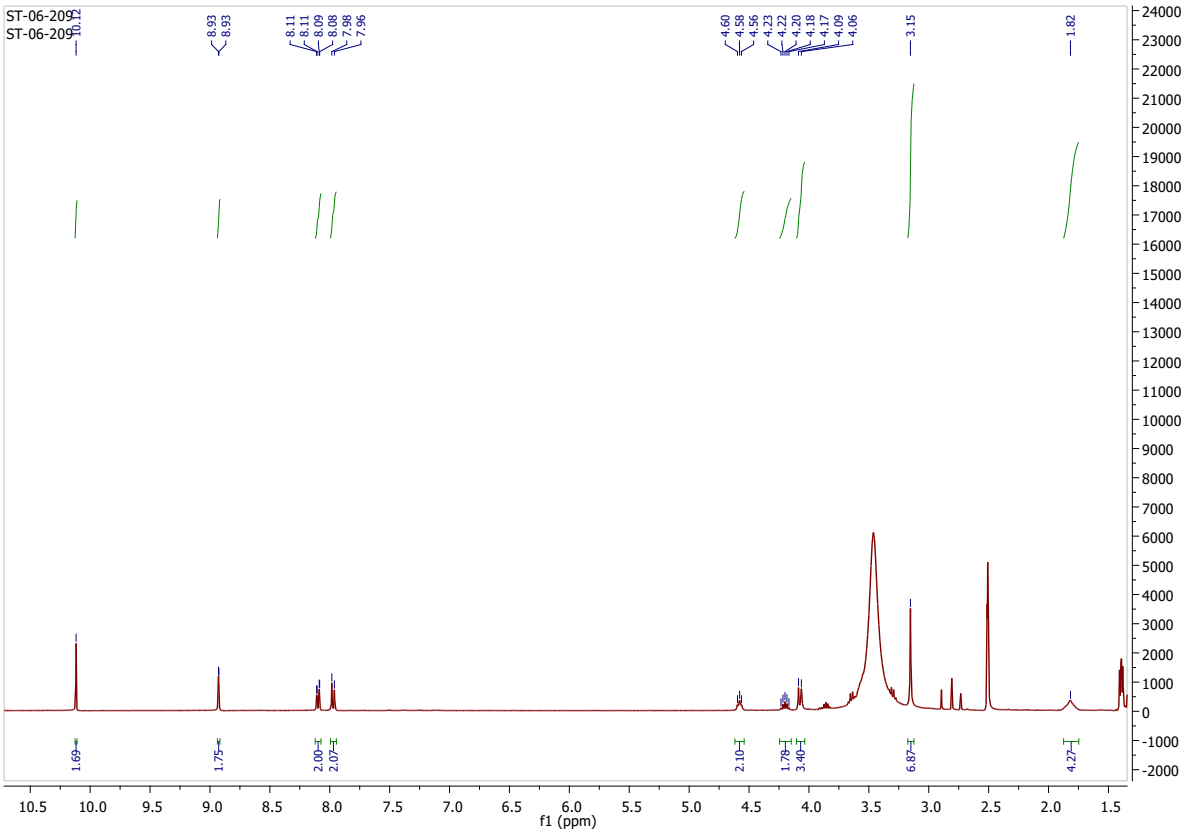
N-(carboxymethyl)-4-(3,6-diformyl-9H-carbazol-9-yl)-N,N-dimethylbutan-1-aminium bromide (6)



5 **6**

To a solution of the ester (1 g,) in CH_3OH (15 ml,) was added 4.0 Equiv. of 1.0 N NaOH. The solution was stirred at r.t for 6 h and neutralized with 4 Equiv. of 1N HCl. The hydrolysis reaction was assumed with 100% yield as monitored on TLC. The reaction mix. was vacuum dried and precipitated with 2 ml of methanol. The product was isolated as brown powdered solid. **H^1 NMR (400MHz, DMSO-d6)**: δ 10.12 (s, 2H), 8.9 (s, 2H), δ 8.10 (d, j = 14Hz,2H), δ 7.97 (d, j = 14Hz, 2H), δ 4.23-4.06 (m,4H), 3.14 (s, 6H), 1.82 (m, 4H). **ESI-HRMS (m/z)** [M^+] Cald. =381.1809, found = 381.1811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8.09
8.08
7.98
7.96
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4.58
4.56
4.23
4.22
4.20
4.18
4.17
4.09
4.06
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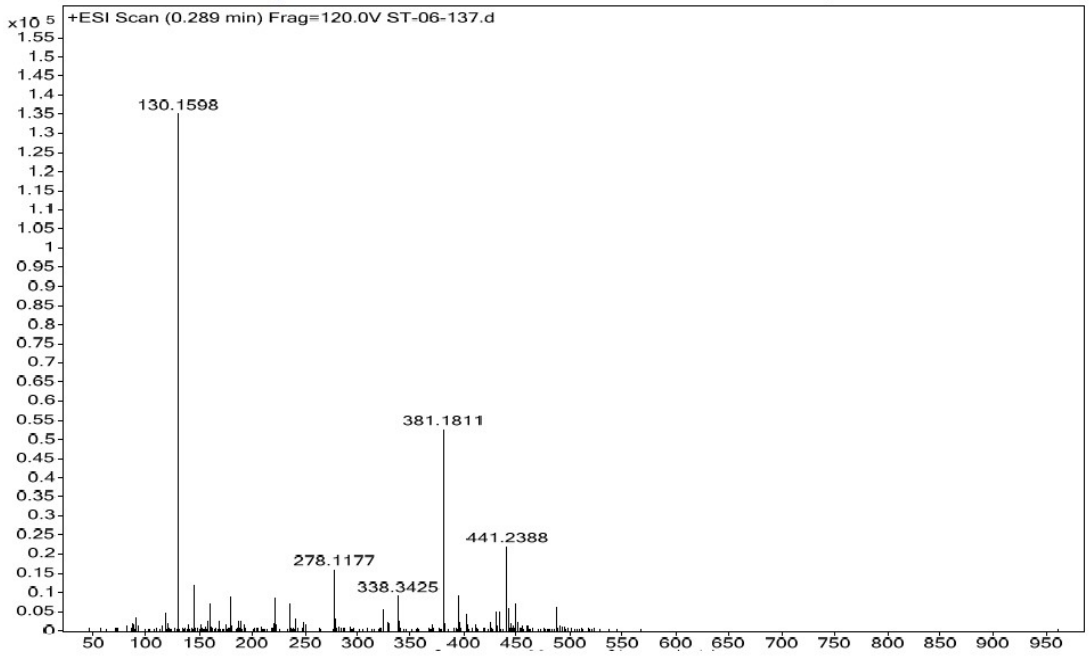
f1 (ppm)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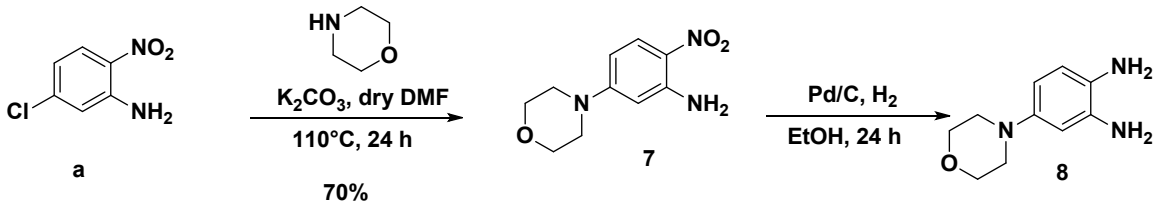
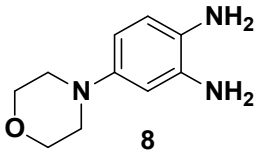
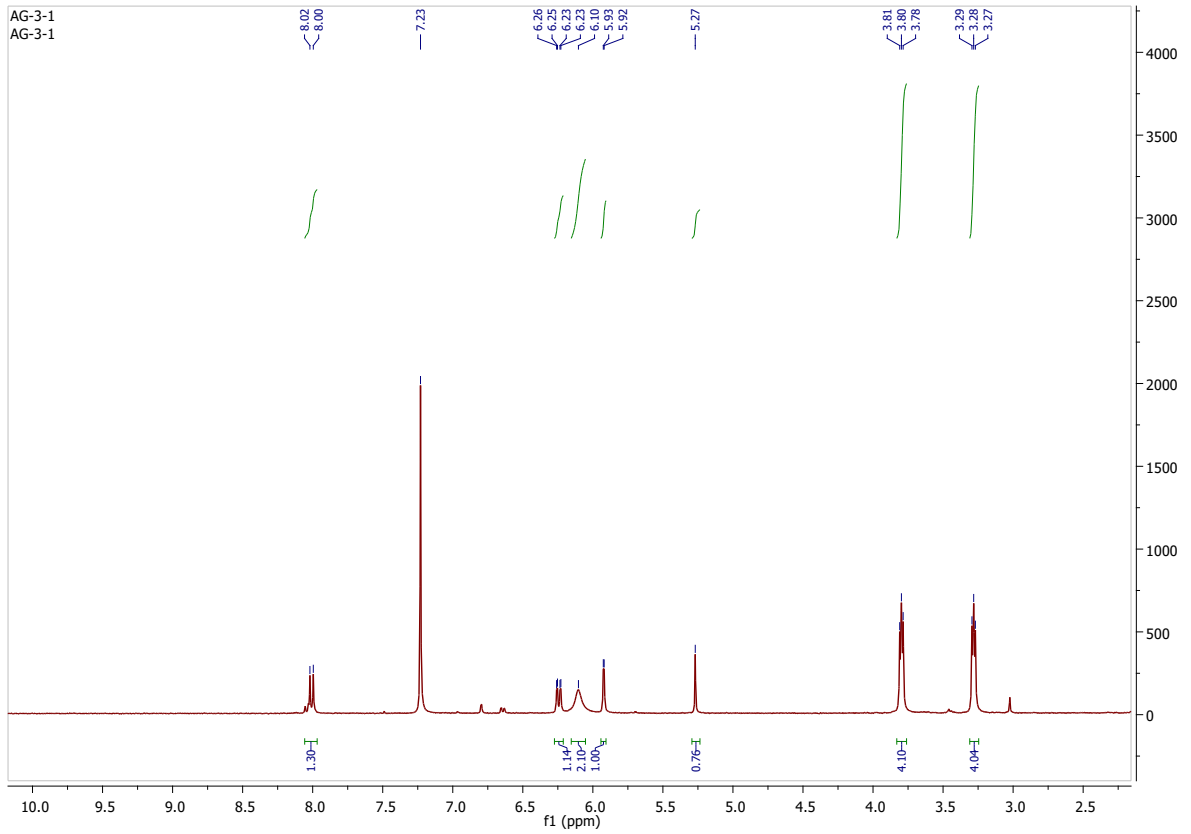
4-morpholinobenzene-1,2-diamine (8)



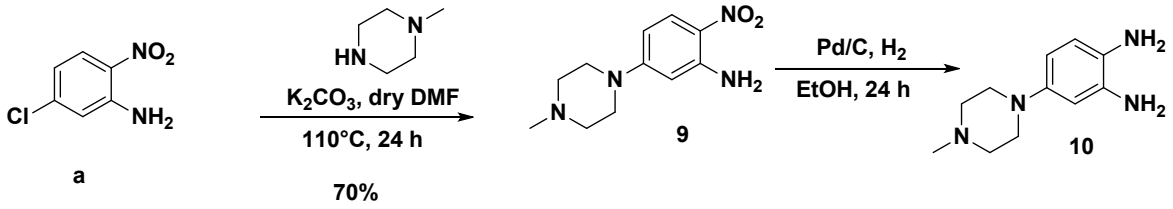
1 g, of 5-Chloro-2-nitroaniline (5.79 mmoles) was taken in 6ml of dry DMF, 2g Morpholine (22.95) and anhydrous K_2CO_3 (8.68 moles) were added. The reaction was allowed to run at 110 °C overnight under inert atmosphere until TLC showed the disappearance of reactant. The crude compound was filtered and washed with water multiple times and concentrated, which led to pure product yellow crystalline solid which was adjudged as pure by TLC (1% MeOH/$CHCl_3$



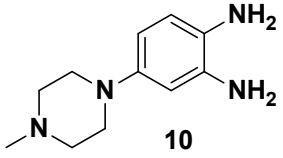
Compound a (344mg) was taken along with 150 mg of Pd/C (10%) in 100 ml ethanol and stirred under an H_2 atmosphere (1 atm pressure) for 18 h. The formation of product was observed by disappearance of the bright yellow colour of the reactant. When the Reaction mixture turned colourless the reaction was assumed to be complete. The reaction mixture was then passed through a Celite bed under nitrogen flow and used for the next reaction without any further purification, as the diamine product was found to be highly unstable.



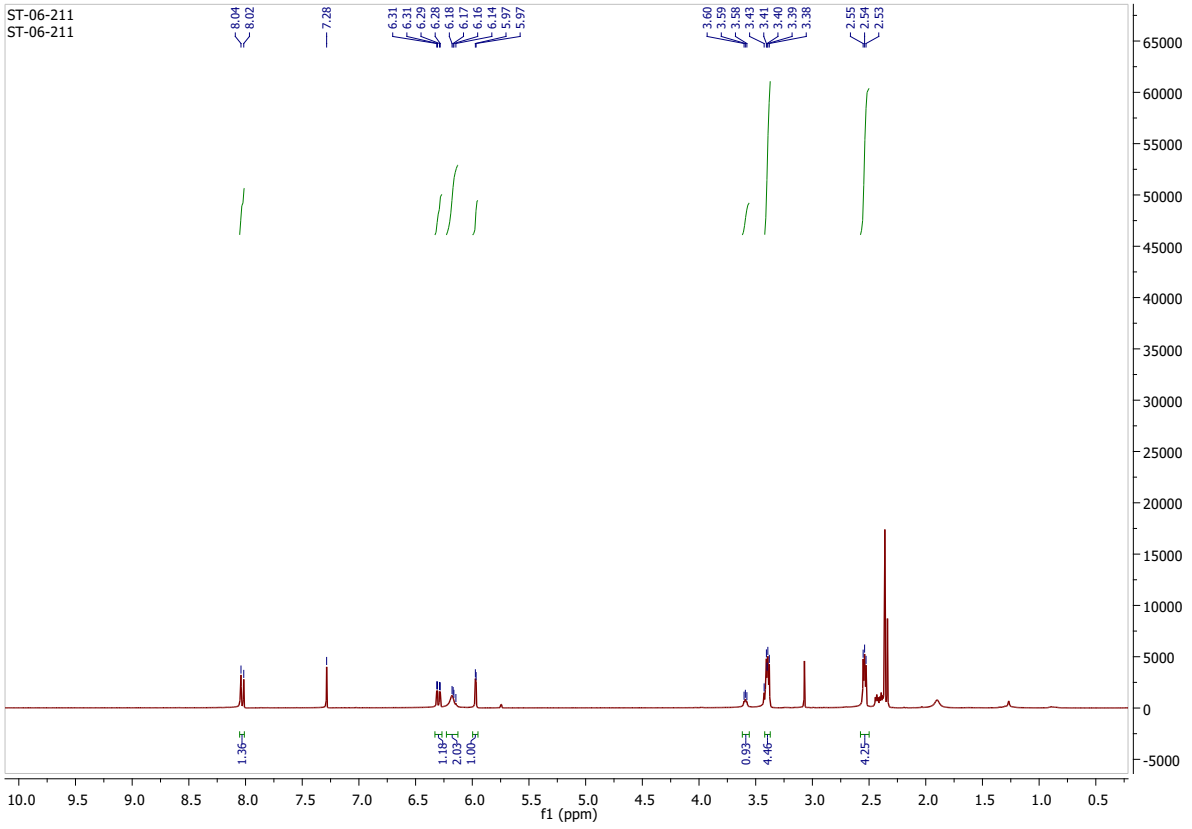
4-(4-methylpiperazin-1-yl)benzene-1,2-diamine. (10)



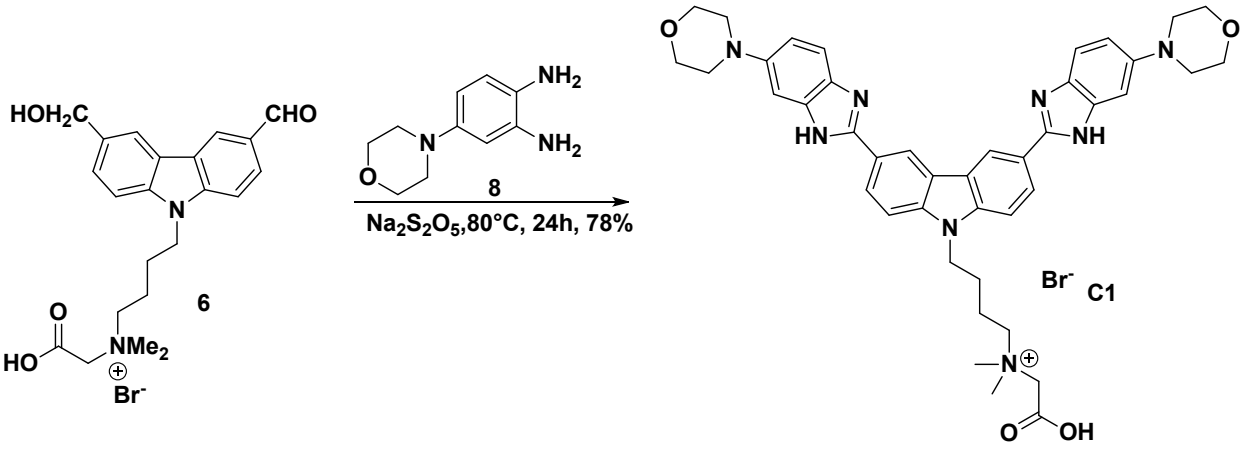
2.5 g, of 5-Chloro-2-nitroaniline (14.5 mmoles) was taken in 6ml of dry DMF, and to that, 4-methylpiperazine (17.4mmoles) and anhydrous K_2CO_3 (21.75 mmol) were added. The reaction was allowed to run at 110 °C overnight under inert atmosphere until TLC showed the disappearance of reactant. The crude compound was filtered and washed with water multiple times and concentrated, which led to pure product yellow crystalline solid which was adjudged as pure by TLC (1% MeOH/$CHCl_3$).



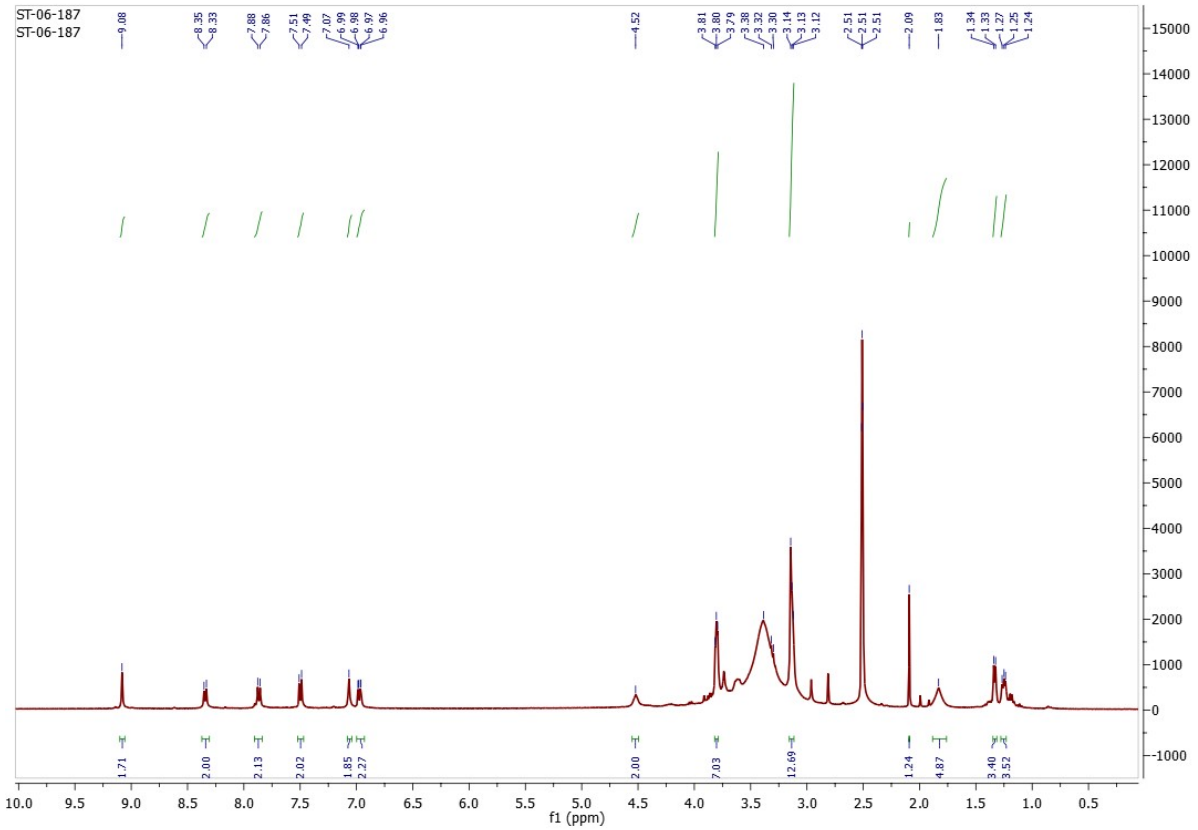
Compound a (206 mg) was taken along with 100 mg of Pd/C (10%) in ethanol and stirred under an H_2 atmosphere (1 atm pressure) for 18 h. The formation of product was observed by disappearance of the bright yellow colour of the reactant. When the Reaction mixture turned colourless the reaction was assumed to be complete. The reaction mixture was then passed through a Celite bed under nitrogen flow and used for the next reaction without any further purification, as the diamine product was found to be highly unstable



4-(3,6-bis(6-morpholino-1H-benzo[d]imidazol-2-yl)-9H-carbazol-9-yl)-N-(carboxymethyl)-N,N-dimethylbutan-1-aminium bromide (C1)



As soon as the compound 8 (200mg, 0.897mmoles) was synthesized it was taken as such without further purification with 180 mg (0.39 mmol) of compound 6 in 30 mL of ethanol. To that, 74mg (0.39 mmol) of $Na_2S_2O_5$ dissolved in 1 mL of water was added and refluxed for 12 h until the reactants were consumed as indicated by TLC. Then, the reaction mixture was cooled to room temperature and filtered. The supernatant was evaporated and purified by repetitive precipitation from a methanol/ethyl acetate mixture, until the product was identified as pure, resulting in a dark brown compound (205mg, 65.28%). **H^1 (400MHz, DMSO-d_6)** δ 9.08 (s,2H), 8.35-8.33 (j=8Hz, d, 2H), 7.87 (j=8Hz, d, 2H), 7.50 (j=8Hz, d, 2H), 6.97, (dd=12 Hz, 2H, 4.52 (s,2H), 3.81-3.79 (m,7H), 3.14-3.12 (m,12H), 2.09 (s, 1H), 1.83 (br s, 4H), 1.34-1.33 (s, j=4Hz, 3H) 1.27-1.24 (m,3H). **ESI-HRMS (m/z)** [m/z] Cald. = 727.3715 found=727.3677



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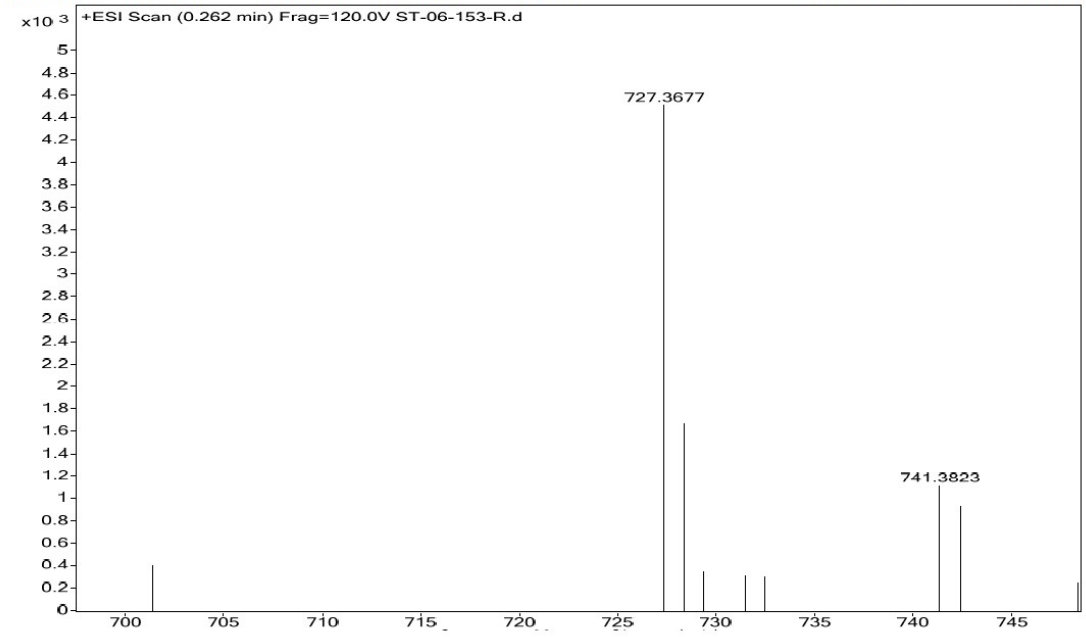
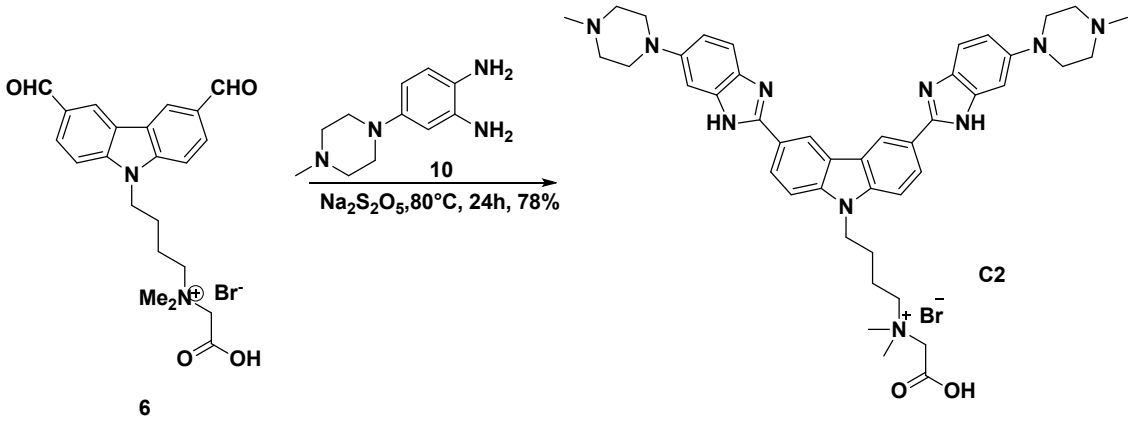
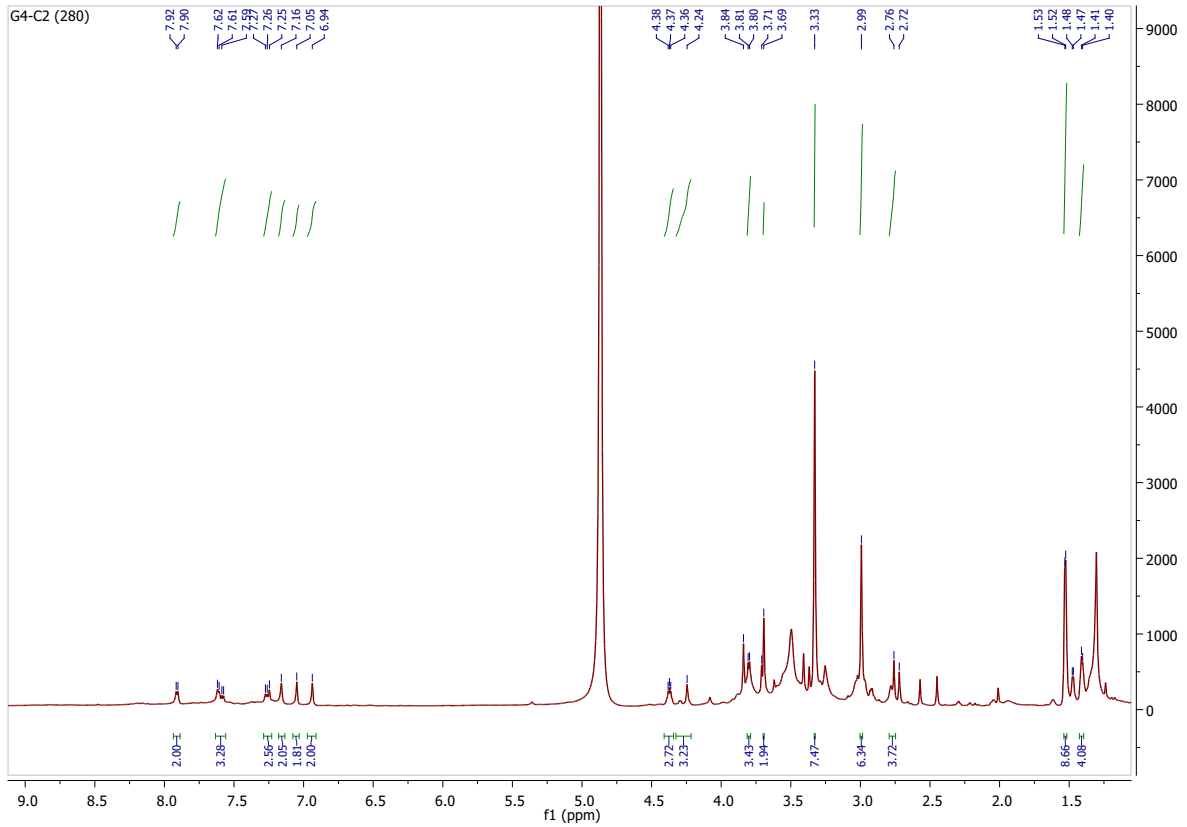


4-(3,6-bis(6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazol-2-yl)-9H-carbazol-9-yl)-N-(carboxymethyl)-N,N-dimethylbutan-1-aminium bromide (C2)



As soon as the compound 10 (111mg, 0.5 mmoles) was synthesized it was taken as such without further purification with 100 mg (0.21 mmol) of compound 6 in 30 mL of ethanol. To that, 40mg (0.21 mmol) of $Na_2S_2O_5$ dissolved in 1 mL of water was added and refluxed for 12 h until the reactants were consumed as indicated by TLC. Then, the reaction mixture was cooled to room temperature and filtered. The supernatant was evaporated and purified by repetitive precipitation from a methanol/ethyl acetate mixture, until the product was identified as pure, by TLC (150mg, 74%). **H^1 (700MHz CD_3OD):** δ7.92(d, j=14Hz, 2H), 7.62-7.67 (m, 3H), 7.25-7.27 (m, 2H), 7.16 (s, 2H), 7.05 (s,2H), 6.94 (s,2H), 4.37(m, 3H), 4.24 (m, 2H), 3.81 (m,3H), 3.69 (m, 2H), 3.33 (s,7H) 2.99 (s, 6H), 3.72 (m, 3H), 1.52 (m,8H), 1.41 (4H)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7.25
7.16
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4.24
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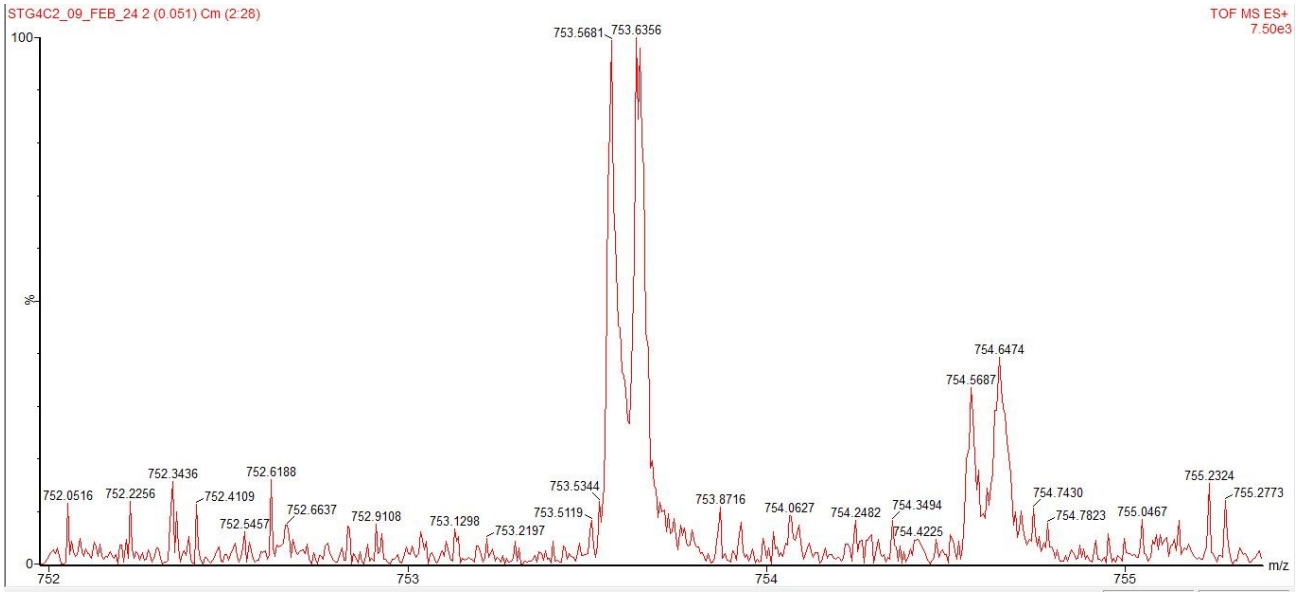
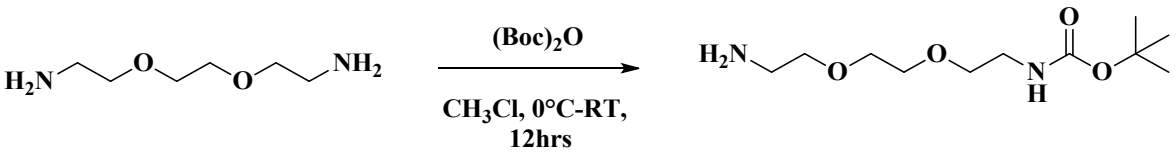
3.81
3.80
3.71
3.69
3.33
2.99
2.76
2.72
1.53
1.52
1.48
1.47
1.41
1.40
2.00
3.28
2.56
2.05
1.81
2.00
2.72
3.23
3.43
1.94
7.47
6.34
3.72
8.66
4.08
f1 (ppm)

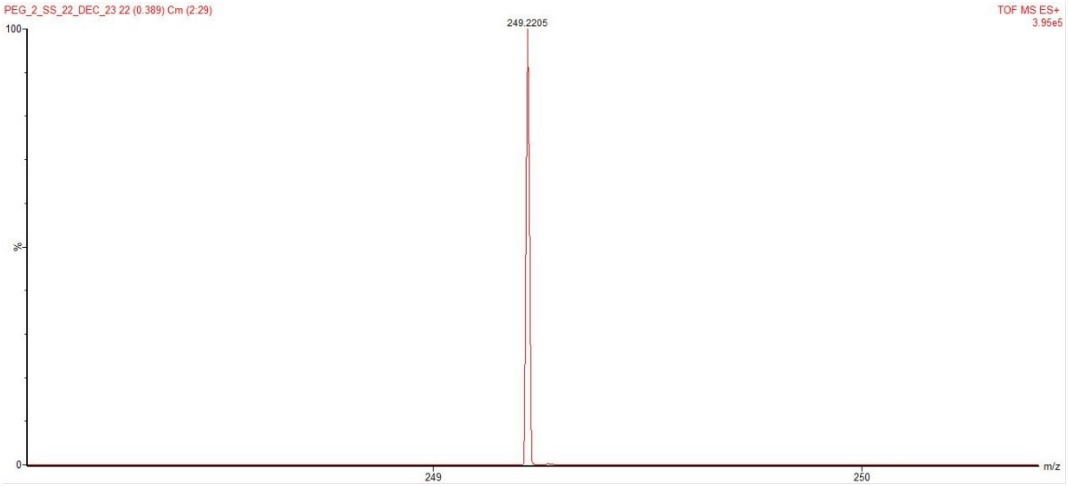

STG4C2_09_FEB_24 2 (0.051) Cm (2:28)
TOF MS ES+
7.50e3
100
%
0
753.5681
753.6356
754.6474
754.5687
752.3436
752.6188
752.0516
752.2256
752.4109
753.5344
752.6637
753.8716
754.0627
754.2482
754.3494
754.7430
755.2324
755.2773
752.5457
752.9108
753.1298
753.5119
753.2197
754.4225
754.7823
755.0467
752
753
754
755
m/z

Synthesis of PEG Linker amine

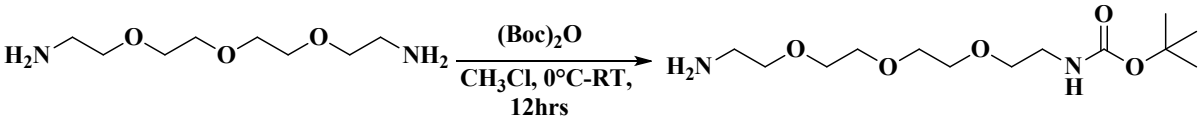
Linker 1: tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate.(13)



To the solution of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethan-1-amine) (3g, 15.6mmoles) in dry CH_2Cl_2 (30 mL), $(Boc)_2O$ (3.6 g) in dry CH_2Cl_2 (120ml) was added dropwise over 0°C for 3h, and then stirred under N_2atmosphere overnight. The reaction mixture was extracted by $CHCl_3$, washed with Na_2CO_3, dried with $MgSO_4$, and evaporated under reduced pressure and the R.M was purified by neutral alumina column Chromatography leading to an oily product (2g,70%)

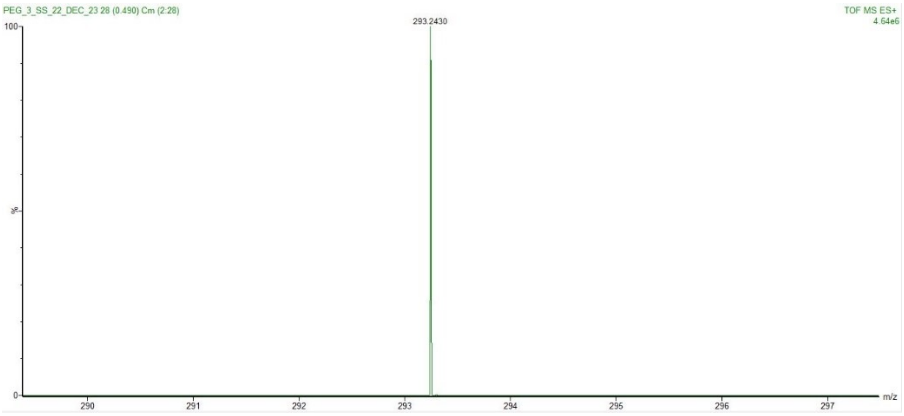


Linker-2: tert-butyl (2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)carbamate (14)



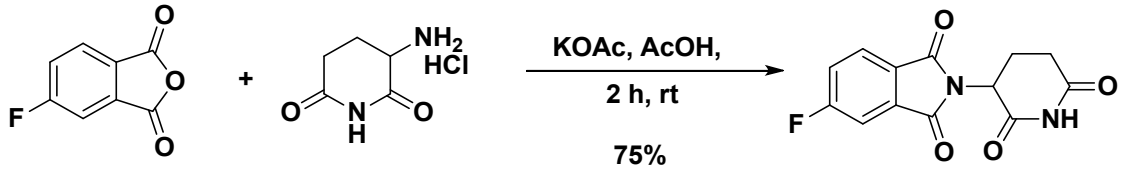
To the solution of the 2,2'-((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethan-1-amine) (3 g, 15.6 mmoles) in dry CH_2Cl_2 (30 mL), $(Boc)_2O$ (3.6 g) in dry CH_2Cl_2 (120 ml) was added dropwise over 0°C for 3h and then stirred under N_2atmosphere overnight. The reaction mixture was

extracted with $CHCl_3$, washed with Na_2CO_3, dried with $MgSO_4$, and evaporated under reduced pressure to obtain an oily product (2 g, 70%).

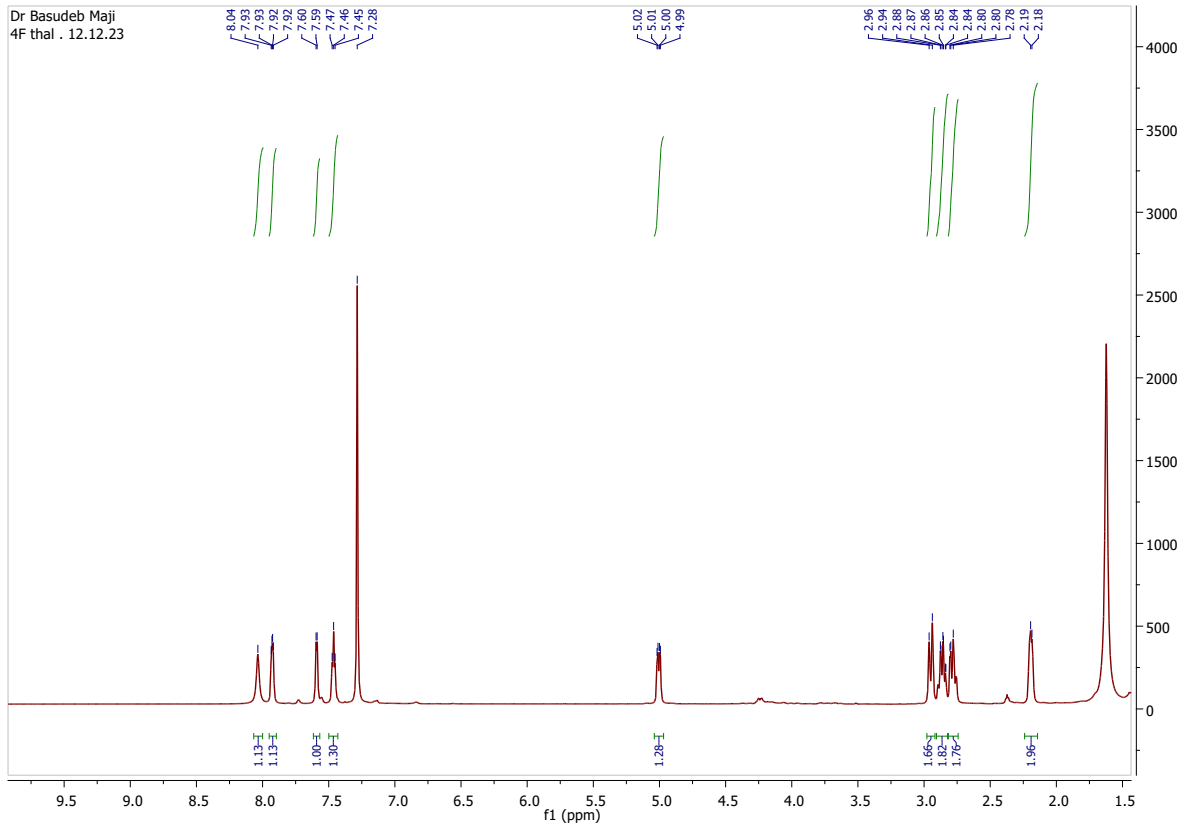


E. Synthesis of (E3 -ligase) CRBN binding moiety.

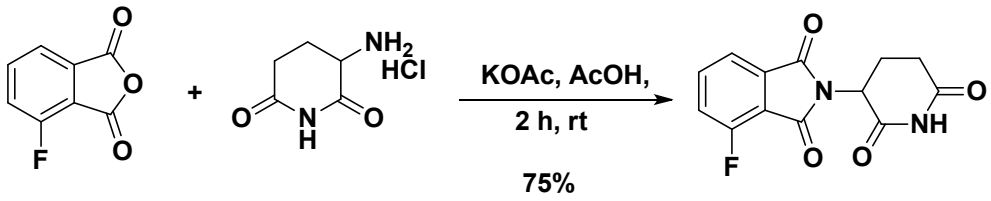
5F thalidomide 2-(2,6-dioxopiperidin-3-yl)-5-fluoroisoindoline-1,3-dione. (15)



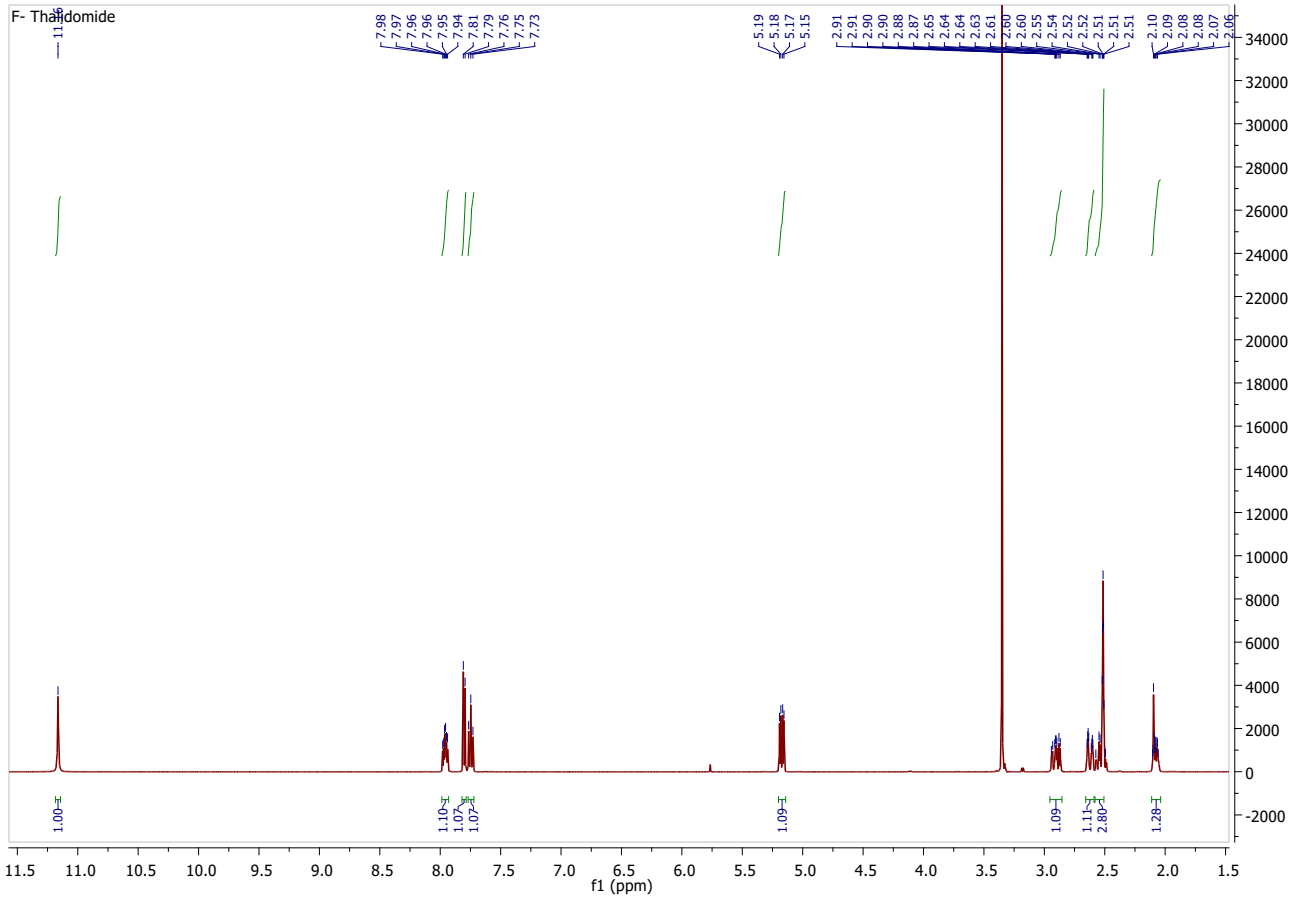
To a round bottom, flask was added 4-fluorophthalic anhydride (1.25 g, 7.5 mmoles) and 3-aminopiperidine-2,6-dione hydrochloride (0.82 g, 5 mmoles). A solution of sodium acetate (0.50 g,6.0 mmoles) in 20 mL glacial acetic acid was added and the dark solution was refluxed for 4 h. After cooling it was poured onto H_2O (100 mL) and the purple solid formed was collected, washed with H_2O (3 × 5 mL) and petroleum ether (3 × 5 mL), and was further dried in a vacuum to give the grey solid as the product **^{1}H NMR (700MHz, $CDCl_3$):** δ 8.06 (s, 1H), 7.96-7.93 (d, 1H), 7.60 (d, 1H), 7.59.5-7.28 (m, 1H), 5.02-4.99 (m,1H), 2.94 (d J= 14 Hz), 2.87-2.84 (m, 2H), 2.80-2.78 (m, 1H).



2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (16)

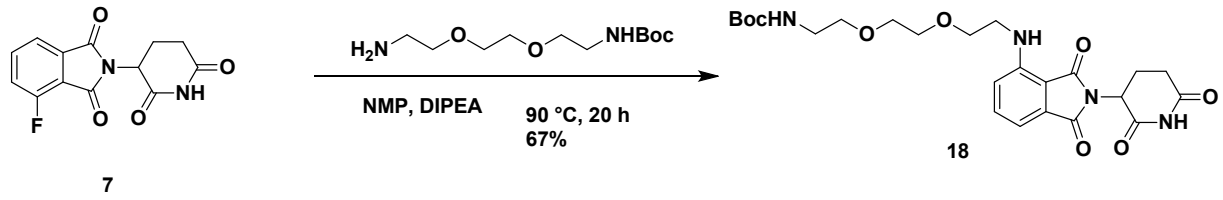


To a round bottom flask, was added 4-fluorophthalic anhydride (1.25 g, 7.5 mmol) and 3-aminopiperidine-2,6-dione hydrochloride (0.82 g, 5 mmol). A solution of sodium acetate (0.50 g,6.0 mmol) in 20 mL glacial acetic acid was added and the dark solution was refluxed for 4 h. After cooling it was poured onto H_2O (100 mL) and the purple solid formed was collected, washed with H_2O (3 × 5 mL) and petroleum ether (3 × 5 mL), and was further dried in a vacuum to give the desired product. **^{1}H NMR (400MHz, DMSO-d$_6$):** δ 11.16 (s, 1H), 7.98-7.94 (m, 1H), 7.79-7.81 (d, j = 8 Hz 1H), 7.76-7.73 (m, 1H) 5.17-5.17 (m, 1H), 2.94-2.87 (m, 1H), 2.65-2.60 (m, 1H), 2.57-2.54(m,2H), 2.10-2.06 (m,1H). **ESI-HRMS (m/z)**



D. Conjugation of the linker and florothalidomide conjugates.

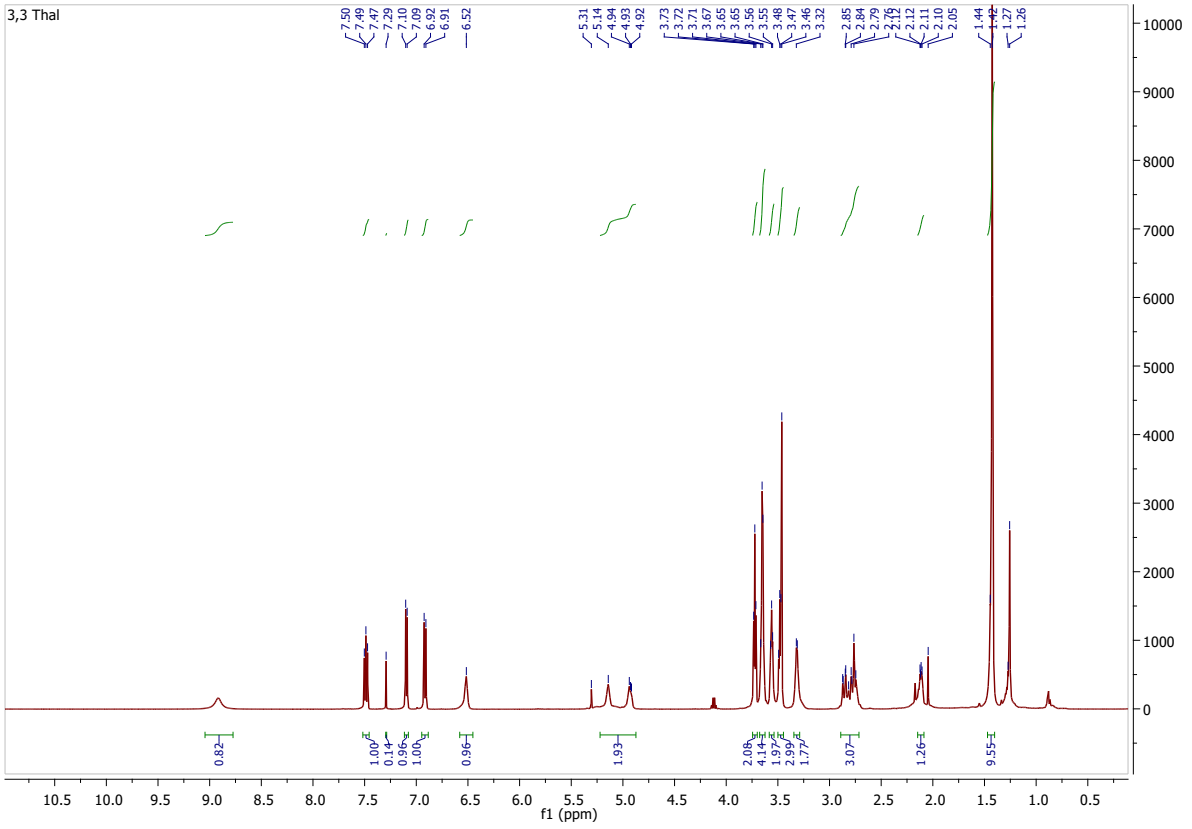
tert-butyl (2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethoxy)ethoxy)ethyl)carbamate (20)



7 18

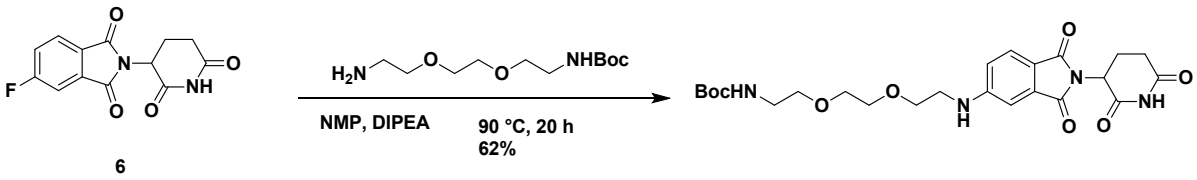
To the solution of 2-(2,6-dioxopiperidin-3-yl)-5-fluoroisoindoline-1,3-dione (300mg, 1.08mmoles) in NMP add DIPEA (0.85ml, 4.96mmoles). The reaction was run at 90°C for 12h. The reaction was cooled to rt, then diluted with EtOAc. And washed with water followed with brine. The products were column purified by a silica gel column (100-200 mesh), 2%MeOH/$CHCl_3$ as yellow fluorescent solids. **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.49 (t, 1H), 7.10 (d, j = 8Hz, 1H), 6.92 (d, J= 8hz, 1H), 6.52 (br. s, 1H), 3.72 (t, 2H), 3.67-3.65 (m, 4H),

3.56 (t, 2H), 3.49-3.46 (m, 2H), 3.32 (m, 2H), 2.87-2.75 (m, 3H), 2.12-2.10 (m, 1H), 1.41 (s, 9H).

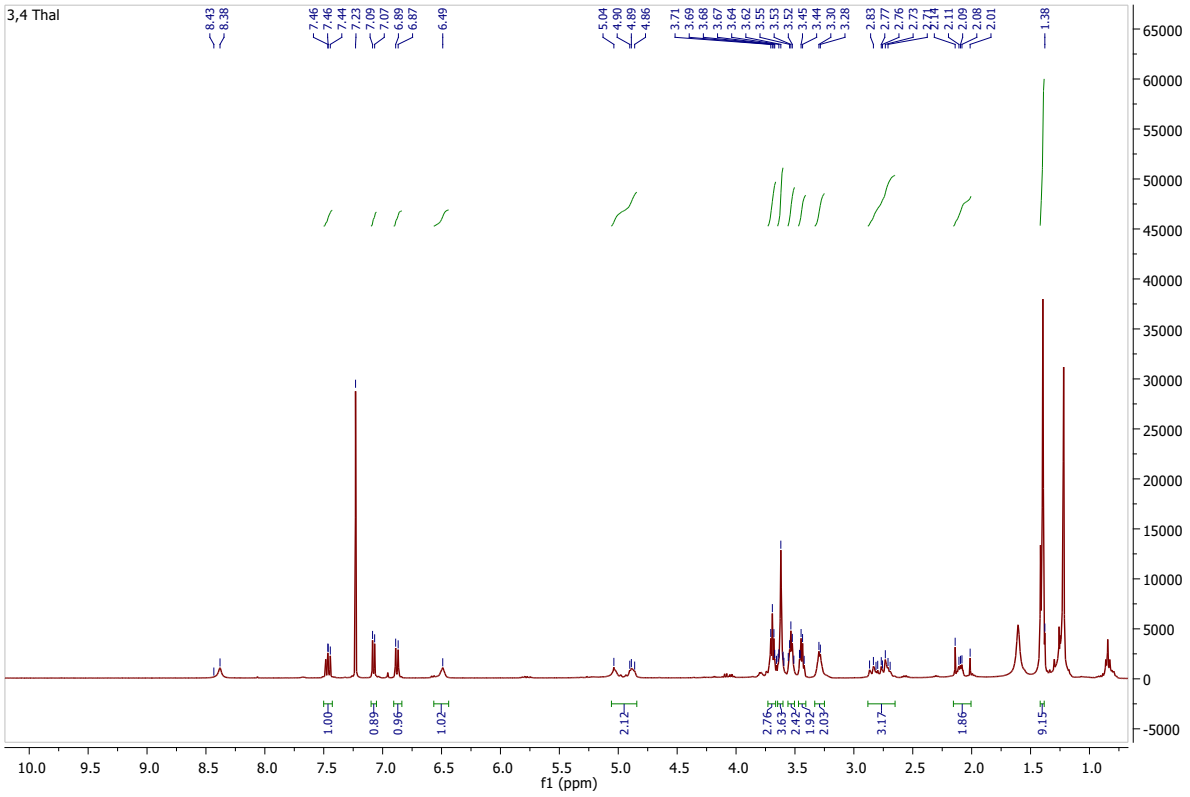


Compounds 18, 19 and 20 were synthesized with the same procedure as reported [1].

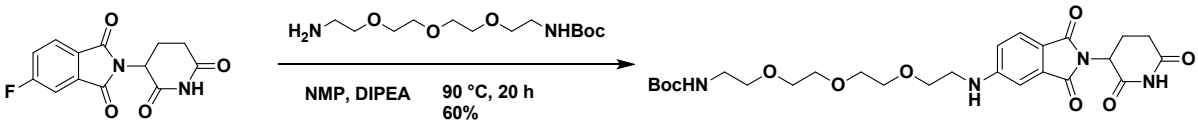
Tert-butyl (2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)ethoxy)ethoxy)ethyl)carbamate (18)



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (t, 1H), 7.09 (d, j = 8Hz, 1H), 6.89 (d, J= 8hz, 1H), 6.49 (br. s, 1H), 5.04-4.86 (m, 2H),3.69 (t, 2H), 3.64-3.60 (m, 4H), 3.56-3.51 (m, 2H), 3.46-3.42 (m, 2H), 3.330 (m, 2H), 2.87-2.09 (m, 3H), 2.14-2.01 (m, 1H), 1.38 (s, 9H).

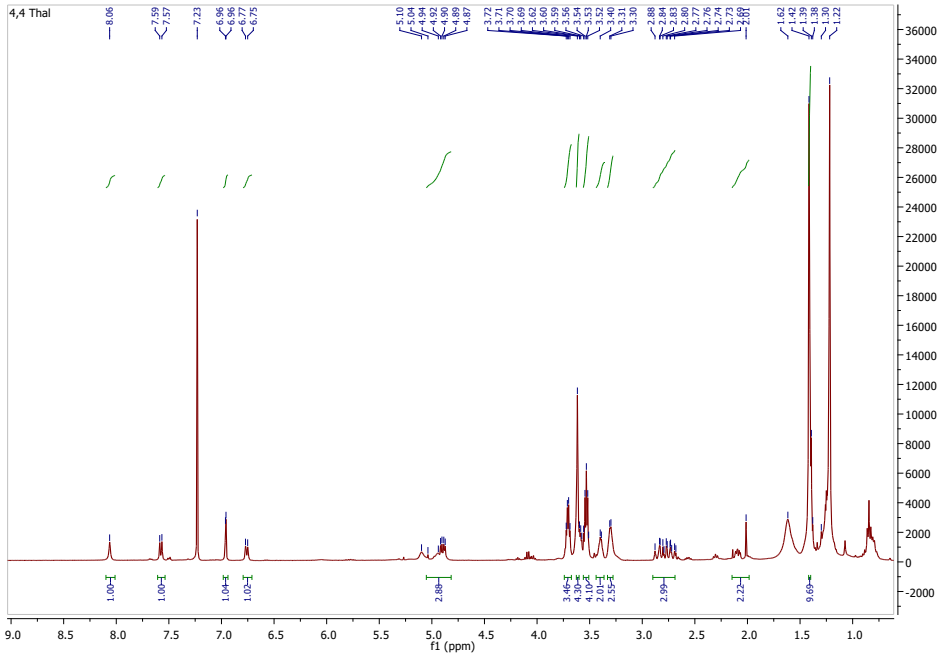


tert-butyl(2-(2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)ethoxy)ethoxy)ethoxy)ethyl)carbamate

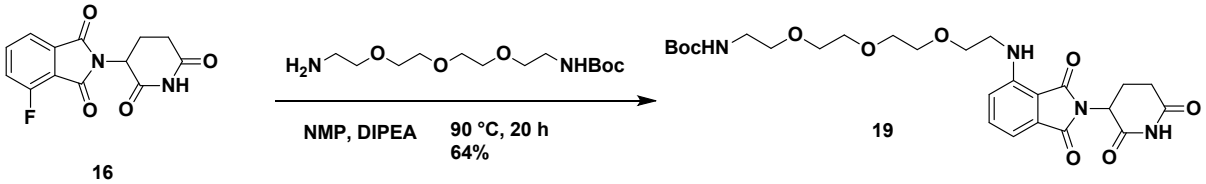


15

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.59 (t, 1H), 7.09 (d, j = 8Hz, 1H), 6.96 (d, J= 8Hz, 1H), 6.77 (br. s, 1H), 5.10-4.87 (m,2H), 3.72-3.69 (m, 4H), 3.56-3.51 (m, 4H), 3.40 (m, 2H), 3.30 (m, 2H), 2.88-2.68 (m, 3H), 2.14-2.01 (m, 1H), 1.42 (s, 9H).



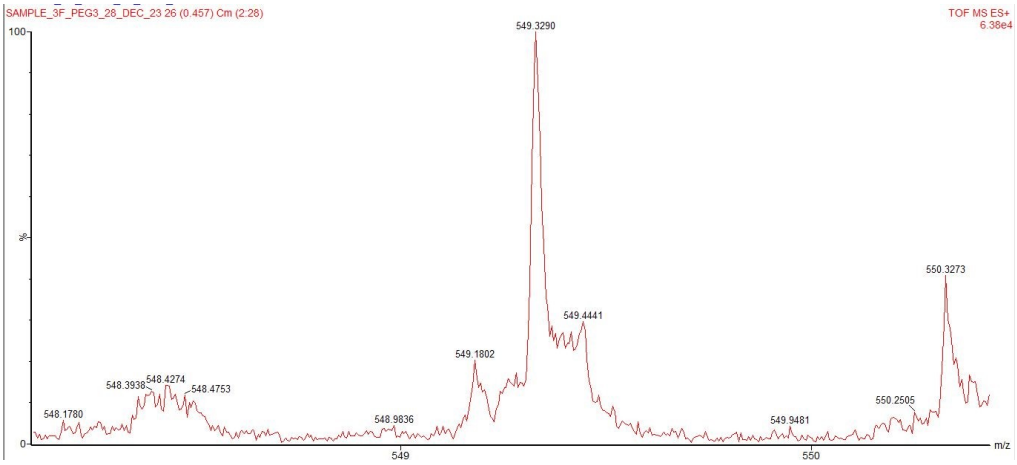
tert-butyl(2-(2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)ethoxy)ethoxy)ethoxy)ethyl)carbamate. (17)



16 19

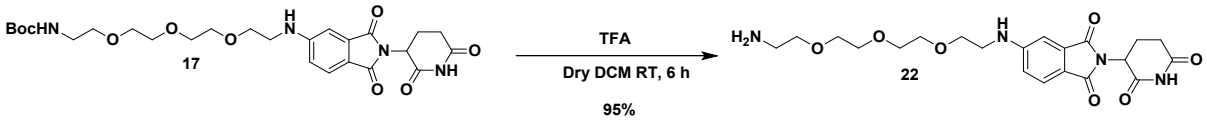
To the solution of 2-(2,6-dioxopiperidin-3-yl)-5-fluoroisoindoline-1,3-dione (300mg, 1.08mmoles) in NMP add DIPEA (0.85ml, 4.96mmoles). The reaction was run at 90°C for 12h. The reaction was cooled to rt, then diluted with EtOAc. And washed with water followed with brine. The product was column purified by a silica gel column (100-200 mesh),

2%MeOH/$CHCl_3$ as yellow fluorescent solids. **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.59 (d, J = 8 Hz, 1H), 6.98 (d, J=8Hz, 1H), 6.77 (dd, J =12, 4 Hz, 1H), 5.27-4.87 (m, 2H), 3.62 (br. s, 2H), 3.52 (t, j = 4Hz, 4H), 3.46 (s, 2H), 3.40-3.36 (m, 3H), 2.88-2.68 (m, 3H), 2.30-2.24 (m, 1H), 1.57 (s, 9H). **ESI-HRMS (m/z)** [m/z] Cald. = 549.2572, found = 549.3290



E. Deprotection of Boc-protected thalidomide linker

5-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (22)

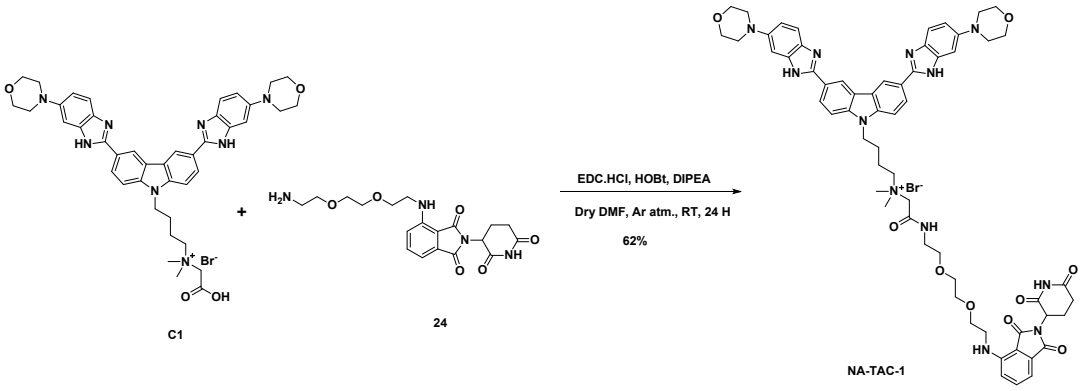


To the solution of 100mg of the Boc-protected compound in Dry DCM, excess TFA was added under inert conditions the R.M. was stirred at room temperature for 6h, TLC was checked, and the product was identified as pure with 95% yield

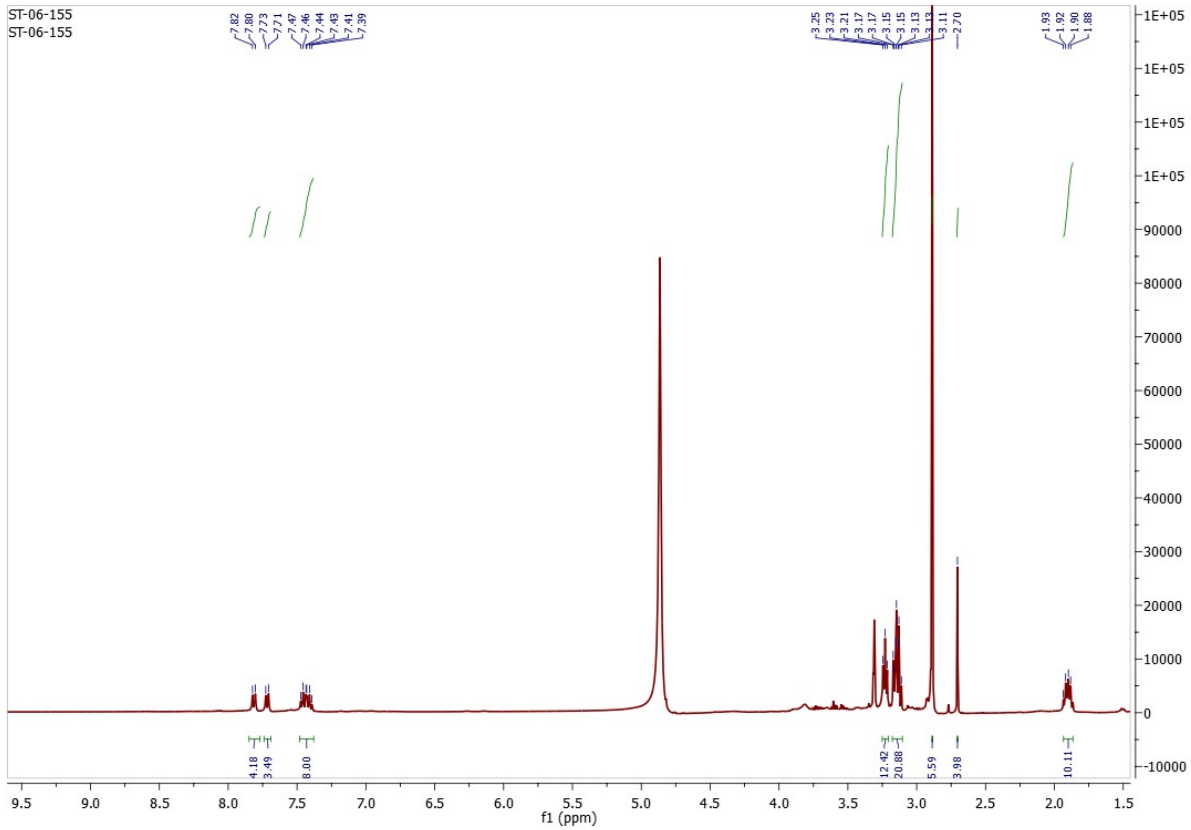
Compounds 23, 24, and 24 were synthesized with the same procedure as reported. (European Journal of Medicinal Chemistry, Volume 218, 2021, 113341, ISSN 0223-5234)

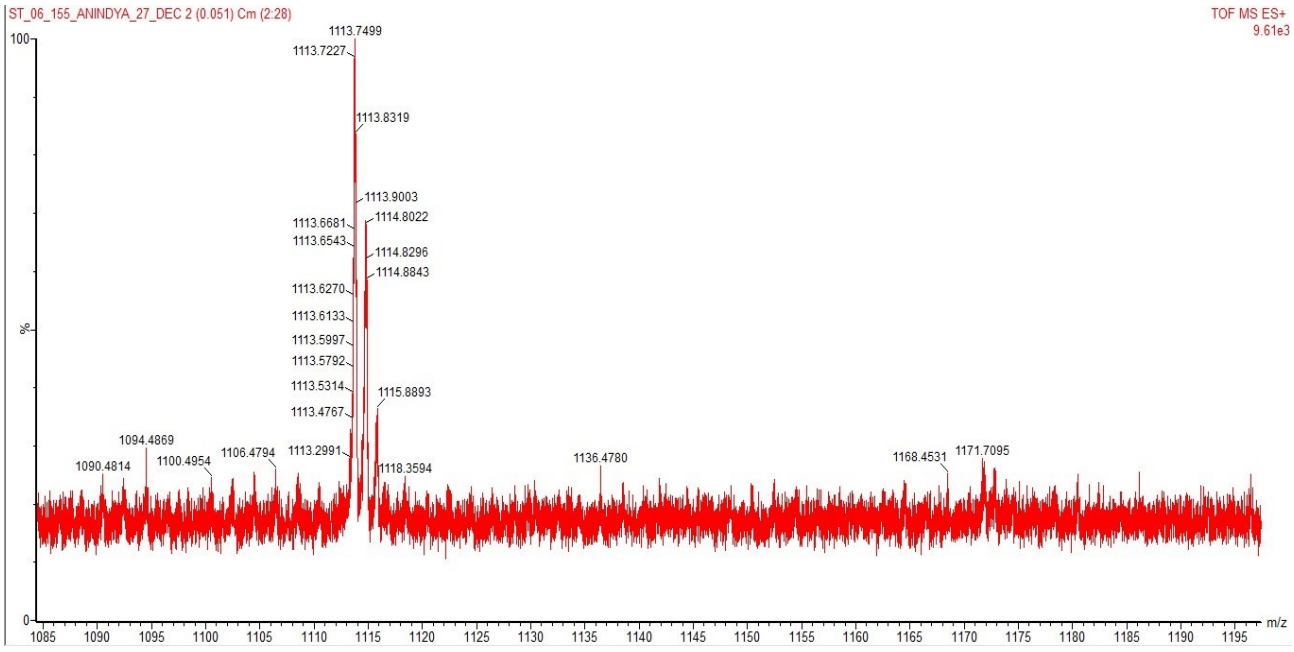
NA-TAC-1 (P1)

4-(3,6-bis(6-morpholino-1H-benzo[d]imidazol-2-yl)-9H-carbazol-9-yl)-N-(2-((2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethoxy)ethoxy)ethyl)amino)-2-oxoethyl)-N,N-dimethylbutan-1-aminium bromide (P1)



To the compound C1 (58.5mg, 0.08mmoles), HOBt (0.16 mmoles), EDC.HCl (0.16mmole), DIPEA (0.8mmoles) was added. The reaction was allowed to stir for 1 hr under Ar atm. To this reaction mixture solution thalidomide linker amine in dry DMF was added. The formation was monitored over TLC by consumption of the amine. The reaction mixture was dried under vacuum; after complete removal of the DMF, the product was isolated by precipitation with DCM. The precipitation was repeated multiple times until the product was identified as pure (yield 61.5%) by TLC. **^{1}H NMR (400 MHz, CD_3OD):** δ 7.84-7.82 (d, j=8Hz,4H), 7.75-7.73 (d, j=8Hz, 3H), 4.49-7.41 (m,8H), 3.27-3.23 (m,12H), 3.19-3.13 (m, 22H), 2.72 (br,s, 4H), 1.96-1.89 (m, 11H). **ESI-HRMS (m/z)** $[M\text{-}Br]^+$ Cald. = 1113.5035, found = 1113.5314


ST-06-155
ST-06-155
f1 (ppm)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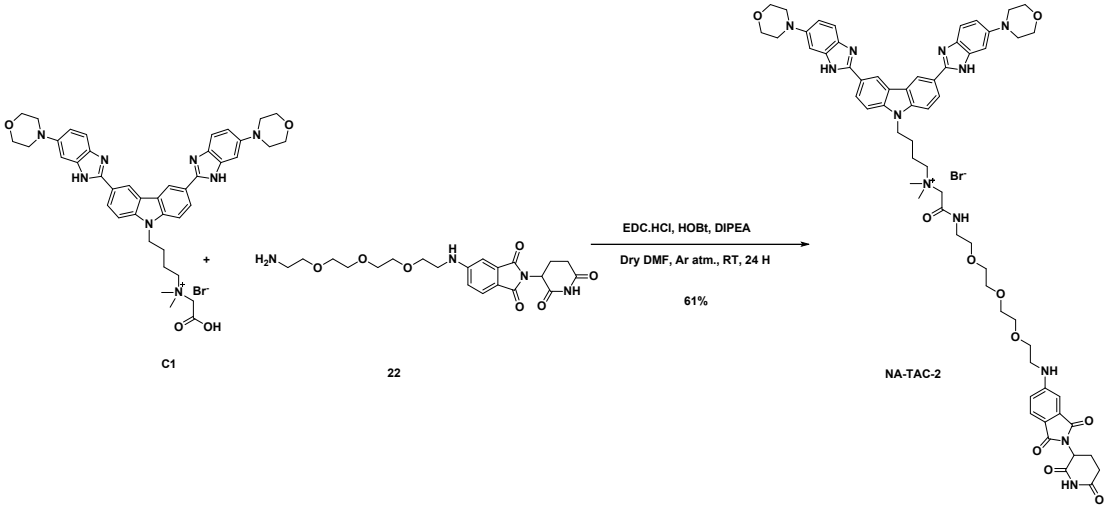
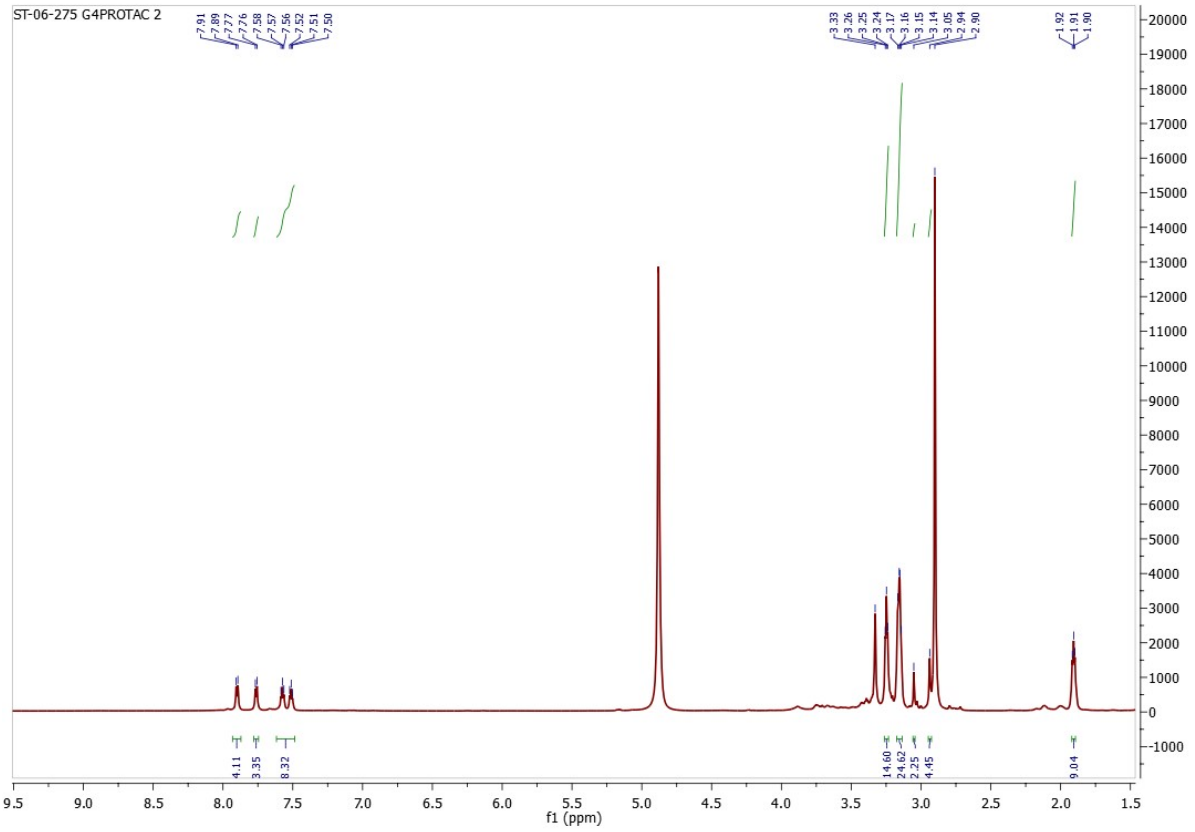
1114.8022
1113.6543
1114.8296
1114.8843
1113.6270
1113.6133
1113.5997
1113.5792
1113.5314
1115.8893
1113.4767
1094.4869
1090.4814
1100.4954
1106.4794
1113.2991
1118.3594
1136.4780
1168.4531
1171.7095
%
m/z

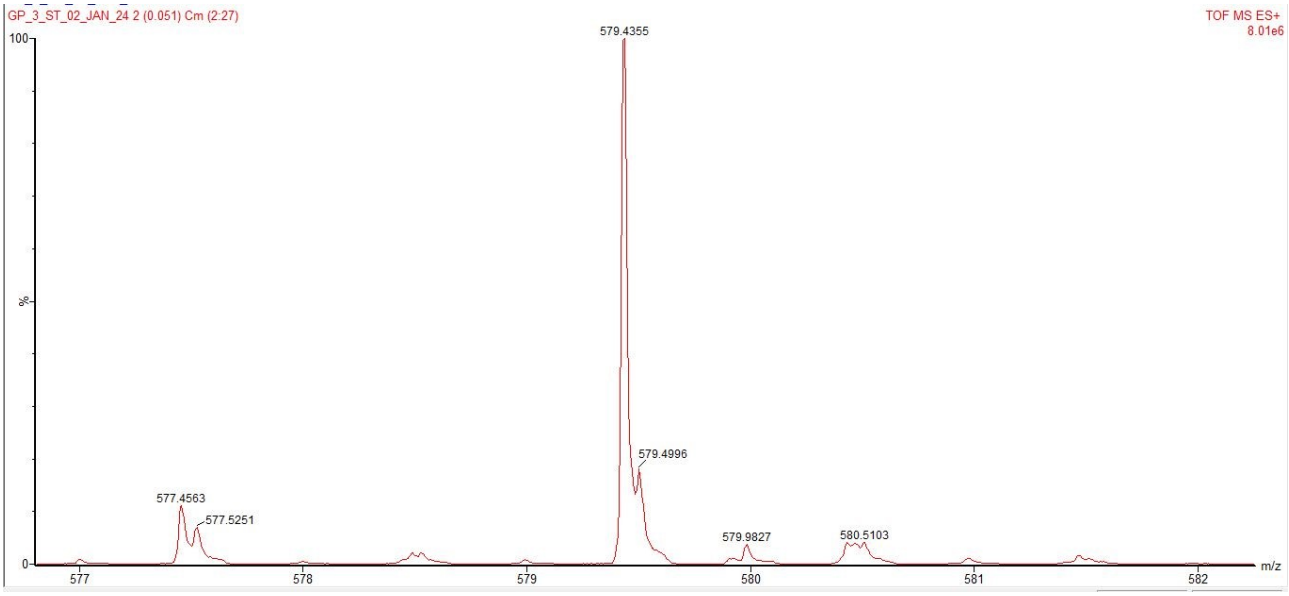
NA-TAC-2 (P2)

N-(4-(3,6-bis(6-morpholino-1H-benzo[d]imidazol-2-yl)-9H-carbazol-9-yl)butyl)-14-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)-N,N-dimethyl-2-oxo-6,9,12-trioxa-3-azatetradecan-1-aminium bromide (P2)



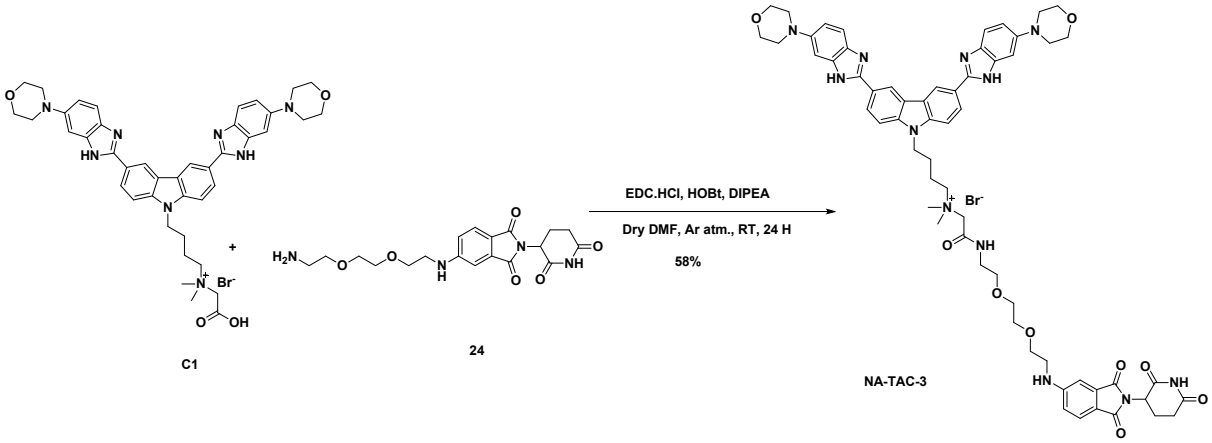
To the compound C1 (30 mg, 0.04 mmoles) in dry DMF, HOBt (0.08 mmoles), EDC.HCl (0.08 mmoles), DIPEA (0.4 mmoles) was added. The reaction was allowed to stir for 1 hr under Ar atm. To this reaction mixture solution of thalidomide linker amine (0.046 mmoles) in dry DMF was added dropwise. The reaction was allowed to run at rt for 24h. The formation of the product was monitored on TLC by consumption of the amine. The reaction mixture was dried under vacuum; after complete removal of the DMF, the product was isolated by precipitating with DCM. The precipitation was repeated multiple times until the product was identified as pure light brown powder (yield 61%) by TLC. **^{1}H NMR (700 MHz, CD_3OD):** δ 7.91-7.89 (d, j=14 Hz, 4H), 7.77-7.76 (d, j=7 Hz, 3H), 7.58-7.50 (m, 8H), 3.26-3.24 (m,14H), 3.17-3.14 (m,24H), 3.05 (br s, 2H), 2.94 (br s, 4H), 1.92-1.90 (m,9H). **ESI-HRMS (m/z)** $[M-Br+H]^{2+}$ (M/2) Cald. =579.2820, found=579.4355



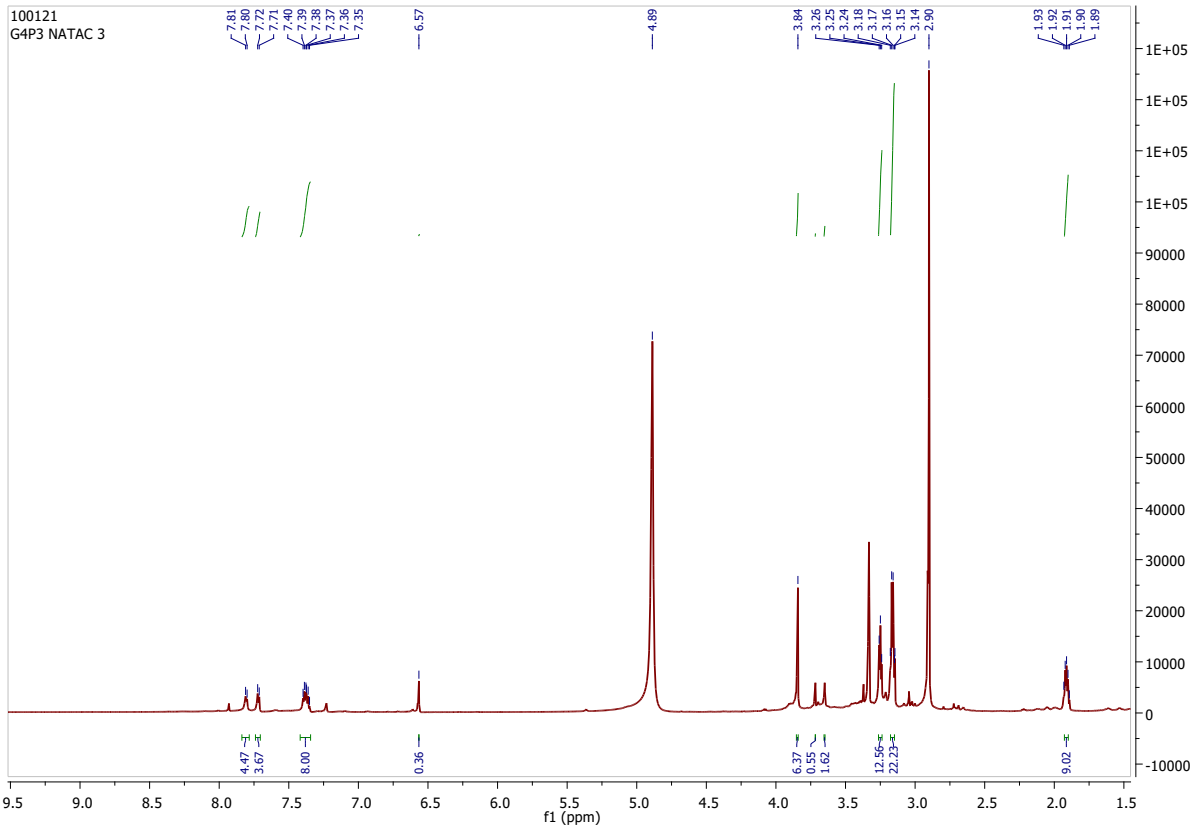


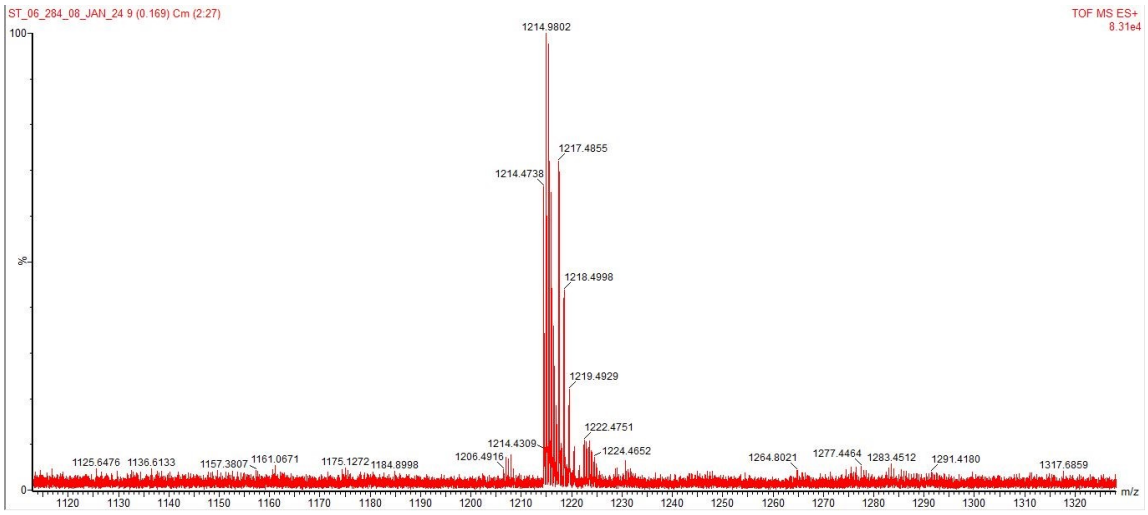
NA-TAC-3 (P3)

4-(3,6-bis(6-morpholino-1H-benzo[d]imidazol-2-yl)-9H-carbazol-9-yl)-N-(2-((2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)ethoxy)ethoxy)ethyl)amino)-2-oxoethyl)-N,N-dimethylbutan-1-aminium bromide (P3)



To the compound C1 (58.5mg, 0.08mmoles), HOBt (0.16 mmoles), EDC.HCl (0.16mmole), DIPEA (0.8mmoles) was added. The reaction was allowed to stir for 1 hr under Ar atm. To this reaction mixture solution of the thalidomide linker amine in dry DMF was added dropwise. The reaction was allowed to stir at RT for 24h.The conversion of reactants into the products was monitored over TLC by consumption of the amine. The reaction mixture was dried under vacuum; after complete removal of the DMF, the product was isolated by precipitation with 10 ml DCM. The precipitation was repeated multiple times until the product was identified as pure (yield 61.5%) by TLC. **^{1}H NMR (700 MHz, CD_3OD):** δ 7.81 (d, j = 7Hz, 4H), 7.72 (d, j = 7Hz, 3H), 7.40 -7.35 (m, 8H), 6.56 (br s, NH) 3.84(brs, 6H), 3.26-3.24 (m,12H), 3.18-.14 (m, 22H), 1.93-1.89 (m, 9H). **ESI-HRMS (m/z)** $[M+Na]^+$ Cald. =1215.4387, found= 1215.4494.


100121
G4P3 NATAC 3
7.81
7.80
7.72
7.71
7.40
7.39
7.38
7.37
7.36
7.35
6.57
4.89
3.84
3.26
3.25
3.24
3.18
3.17
3.16
3.15
3.14
2.90
1.93
1.92
1.91
1.90
1.89
4.47
3.67
8.00
0.36
6.37
0.55
1.62
12.56
22.23
9.02
f1 (ppm)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TOF MS ES+
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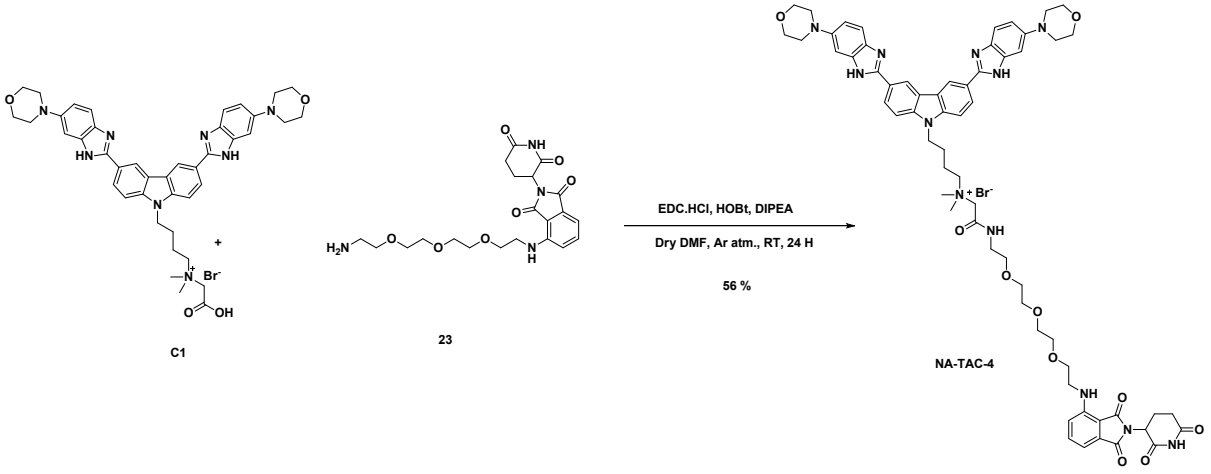
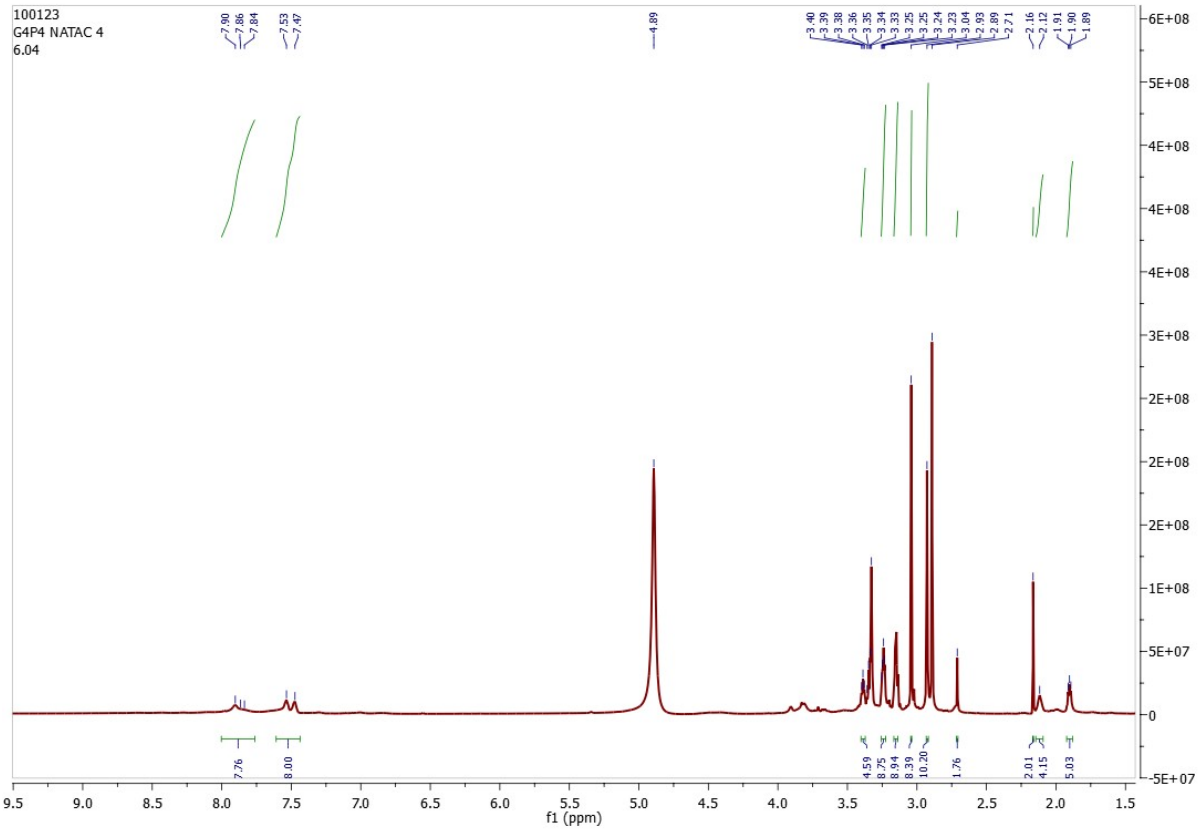
1219.4929
1222.4751
1214.4309
1224.4652
1206.4916
1125.6476
1136.6133
1157.3807
1161.0671
1175.1272
1184.8998
1264.8021
1277.4464
1283.4512
1291.4180
1317.6859
m/z

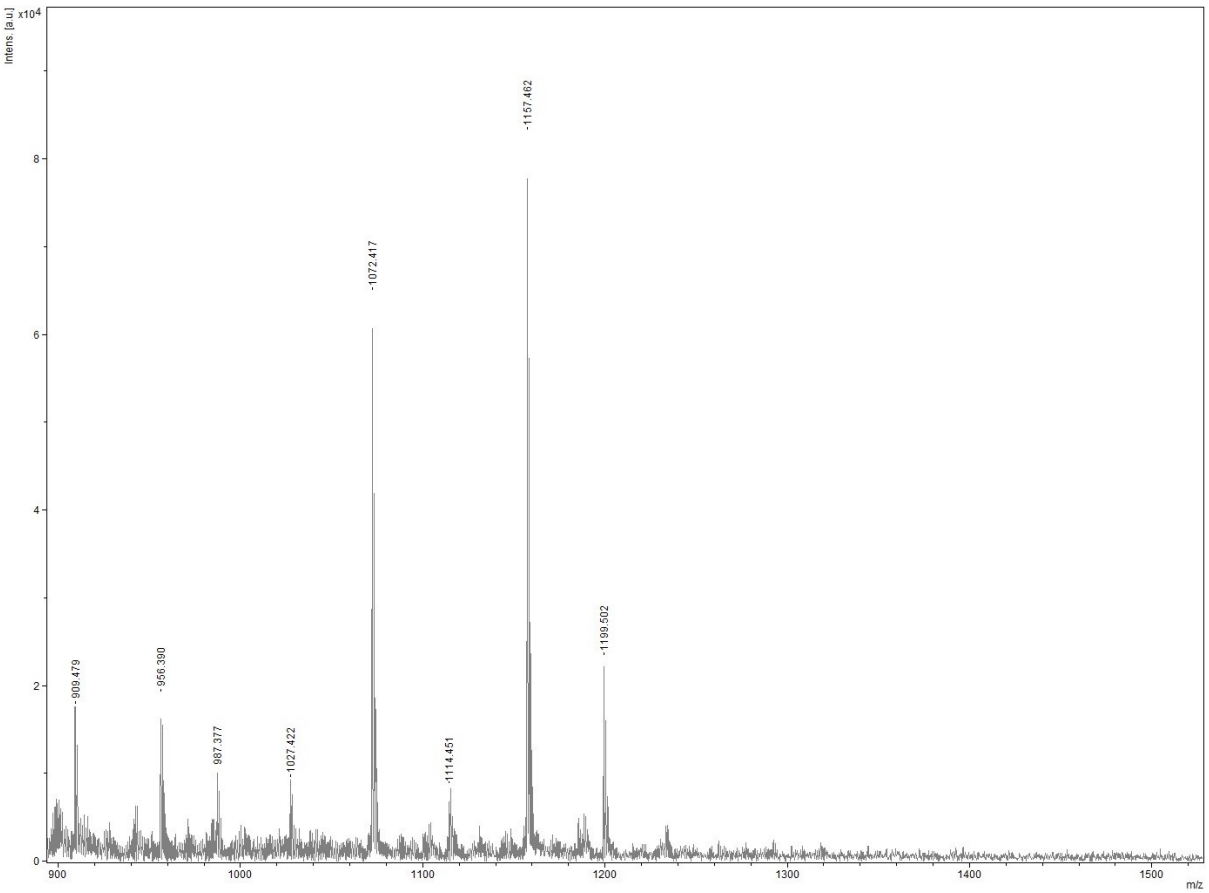
NA-TAC-4 (P4)

N-(4-(3,6-bis(6-morpholino-1H-benzo[d]imidazol-2-yl)-9H-carbazol-9-yl)butyl)-14-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)-N,N-dimethyl-2-oxo-6,9,12-trioxa-3-azatetradecan-1-aminium bromide (P4)



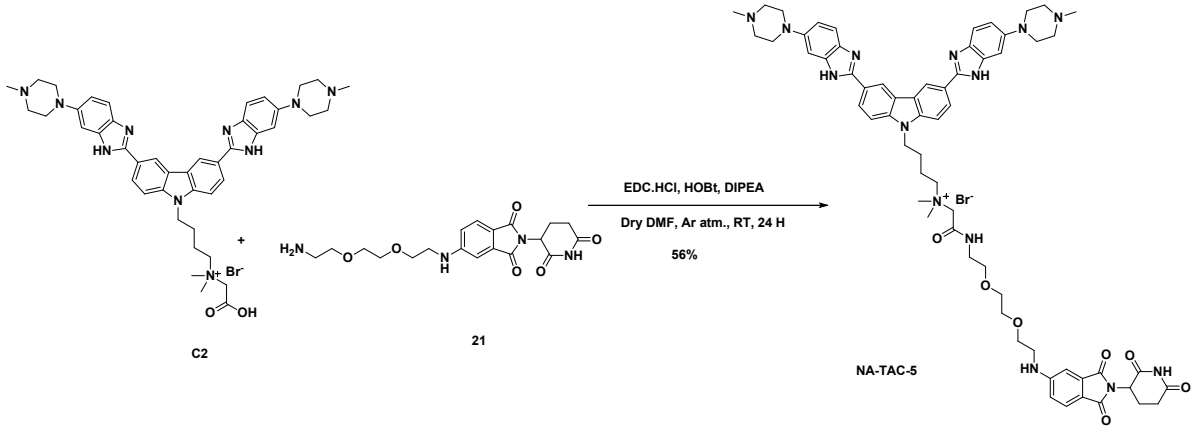
To the compound C1 (50mg, 0.068mmoles), HOBt (0.13 mmoles), EDC.HCl (0.13 mmoles), DIPEA (0.68mmoles) was added. The reaction was allowed to stir for 1 hr under Ar. atm. To this reaction mixture solution of the 33 mg of thalidomide linker amine (0.079 mmoles) in dry DMF was added dropwise. The reaction was allowed to stir at RT for 24h.The conversion of reactants into the products was monitored over TLC by consumption of the amine. The reaction mixture was dried under vacuum; after complete removal of the DMF, the product was isolated by precipitation with DCM. The precipitation was repeated multiple times until the product was identified as pure (yield 53%).**^{1}H NMR (700 MHz, CD_3OD):** δ 7.90 (m, 7H), 7.53-7.47 (m, 8H), 3.40-3.38 (t, j = 7Hz, 4H), 3.25-3.23 (m, 8H), 3.04 (br. s, 8H), 2.93 (br. s, 10H),2.16 (s, 2H), 2.12 (br. s, 8H), 1.76 (s, 1H), 1.91-1.89 (m, 5H). **MALDI-TOF/MS $[M-Br]^+$ (m/z)** Cald. =1157.556, found=1157.462.


100123
G4P4 NATAC 4
6.04
f1 (ppm)

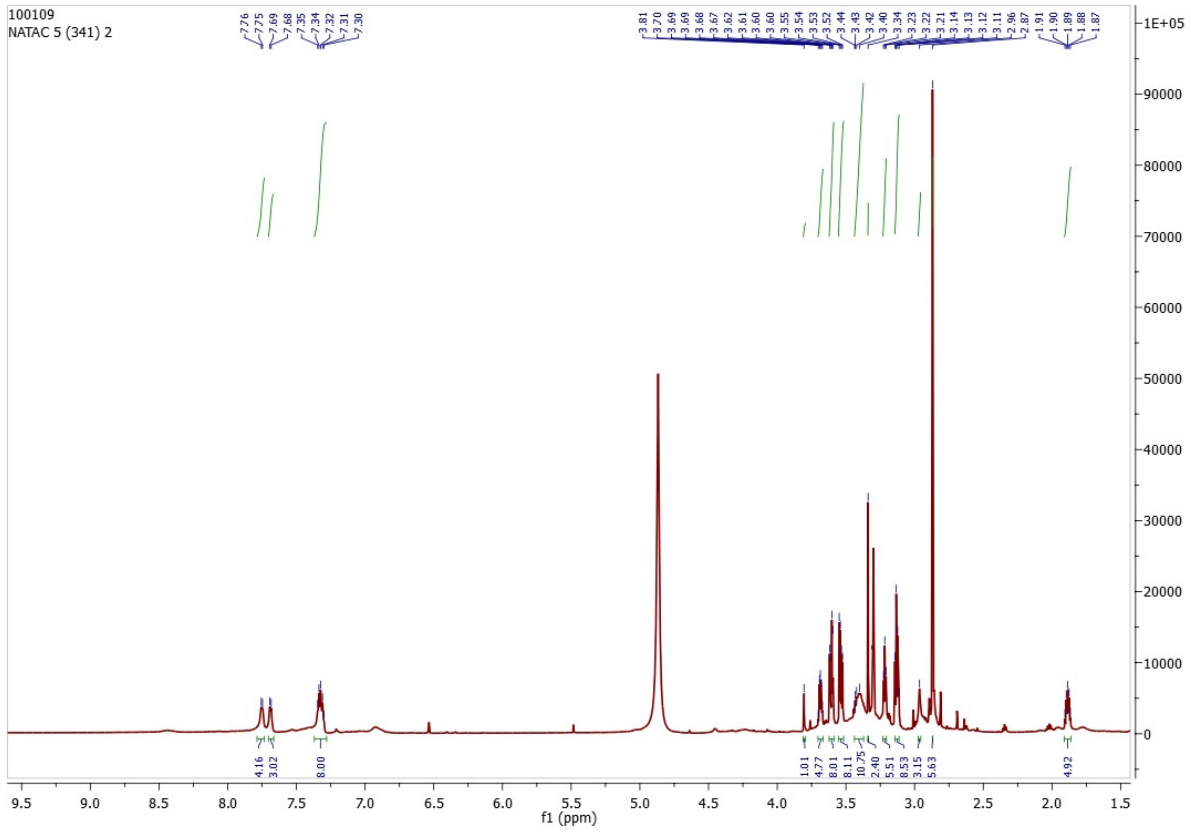

Intens. [a.u.]
x10^4
m/z
1157.462
1072.417
1199.502
909.479
956.390
987.377
1027.422
1114.451

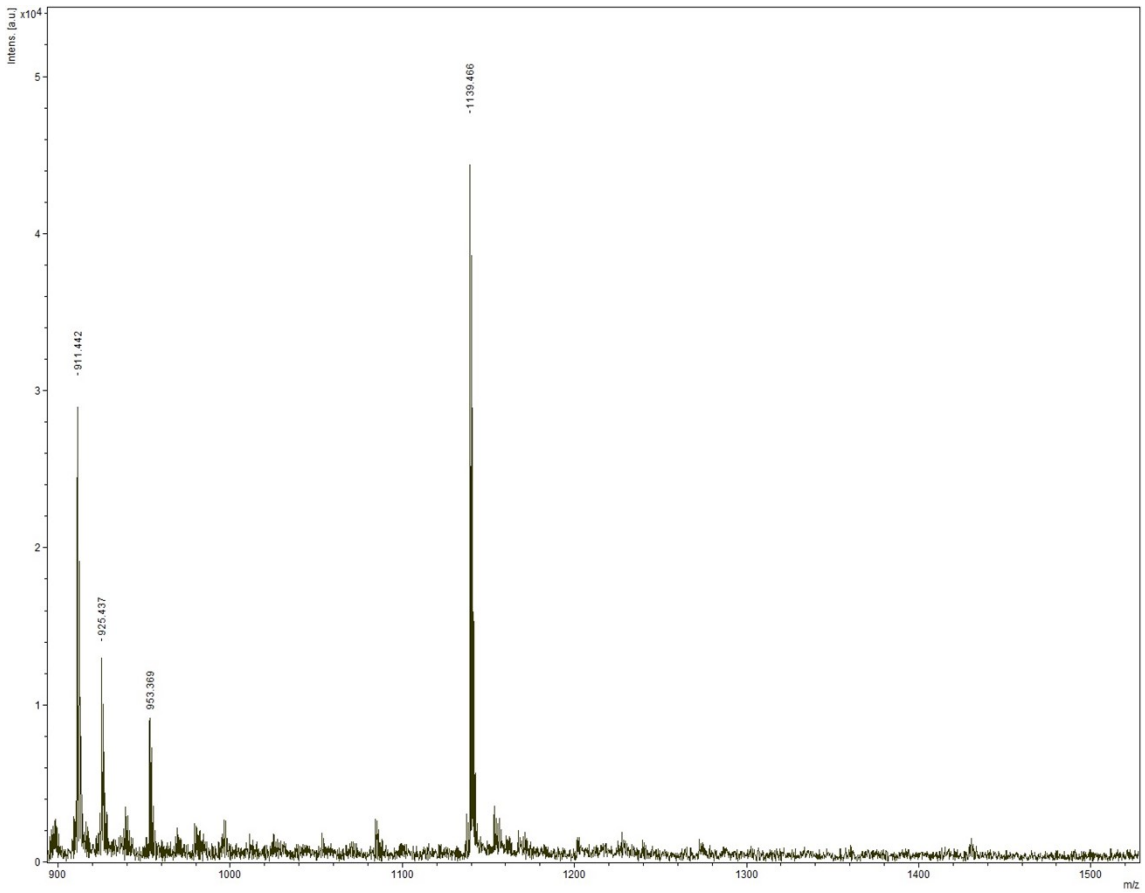
NA-TAC-5 (P5)

4-(3,6-bis(6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazol-2-yl)-9H-carbazol-9-yl)-N-(2-((2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)ethoxy)ethoxy)ethyl)amino)-2-oxoethyl)-N,N-dimethylbutan-1-aminium bromide (P5)



50 mg of the compound C2 (0.076 mmoles) was taken in an oven dried 25 ml round bottom flask having 4 ml of dry DMF. To this HOBt (0.15 mmoles), EDC.HCl (0.15 mmoles), DIPEA (0.76 mmoles) were added. The reaction was allowed to stir for 1 hr under Ar. atm. at rt. To this reaction mixture solution 38 mg of thalidomide linker amine (0.0786 mmoles) in dry DMF was added dropwise. The reaction was allowed to run at this temperature for 16 h. The formation of product was confirmed by TLC by consumption of thalidomide linker amine. The reaction mixture was dried under vacuum to remove DMF completely. The desired product was isolated by precipitation with DCM. The precipitation was repeated multiple times until the product was identified as pure (30 mg, yield 56.3 %) by TLC. **^{1}H NMR (700 MHz, CD_3OD):** δ 7.76 (d, j = 7Hz, 4H), 7.69 (d, j = 7Hz, 3H), 7.35-7.30 (m,8H), 3.81 (s, 1H), 3.70-3.67 (m, 4H), 3.62-3.60 (dd, j = 14Hz, 7Hz, 8H), 3.55-3.52 (dd, j = 14Hz, 7Hz, 8H), 3.44-3.40 (m,10H), 3.34 (s, 2H), 3.23-3.21(t, 5H), 3.14-3.11(m, 8H), 2.96 (br. s, 3H), 1.95-1.87 (m, 5H), **MALDI-TOF/MS $[M-Br]^+$ (m/z)** Cald. =1139.593, found=1139.466.


100109
NATAC 5 (341) 2
f1 (ppm)


Intens. [a.u.]
x10^4
1139.466
911.442
925.437
953.369
m/z

G4 DNA Formation: Hum_21 mer G4 DNA having Sequence 5'-GGGTTAGGGTTAGGGTTAGGG-3' was incubated in a buffer containing 10 mM Tris−HCl (pH 7.4), 0.1 Mm EDTA, 100 Mm KCl and heated at 95 °C for 5 min and cooled slowly to 24 °C over 24 h.

Circular Dichroism Spectroscopy: The CD experiments were performed in a Jasco J-815 CD spectropolarimeter equipped with a Peltier temperature controller in 1 cm quartz cuvette at 20 °C. To the G4 DNA (3 μM) in 10 mM Tris−HCl (pH 7.4), 100 Mm KCl, and 0.1 mM EDTA, 15 μM of all the ligands was added (C1, P1, P2,P3,P4, C2 and P5) and heated at 95 for 5 min followed by slow cooling over 24 h. Next day spectra were recorded with scan rate of 50 nm/min.

DNA Melting Experiment: The melting experiments were performed in a Jasco J-815 CD spectropolarimeter equipped with a Peltier temperature controller. All the samples were prepared in 10 mM Tris−HCl (pH 7.4), 100 Mm KCl, and 0.1 mM EDTA, in which 3 μM 15 μM of all the ligands were added (C1, P1, P2, P3, P4, C2 and P5) and heated at 95 for 5 min followed by slow cooling over 24 h, next day melting temperatures were recorded.

Molecular Docking: To obtain the optimized geometry of the molecules, we carried out first-principles density functional theory (DFT) [2,3] based calculations as implemented in Vienna Ab-initio Simulation Package (VASP) [4,5] associated with the plane wave basis set. Projector-augmented wave (PAW) [6] potentials have been considered for all the constituent elements, e.g., H, C, N, and O. Generalized gradient approximation (GGA) is employed to treat the exchange-correlation function of Perdew-Burke-Ernzerhof (PBE) [7]. For this calculation, the molecule has been placed within a unit cell of dimension 45×45×35 Å3 to diminish the errors attributed to the interactions between the periodically repeating structures. A plane wave cut-off energy of 500 eV has been employed for this calculation. The geometry optimizations are carried out by Γ (gamma) point calculations. To better treat the molecular interactions, we have estimated Van der Waals's total energy of the system using Grimme's DFT-D3 dispersion correction [8], as implemented in VASP. The allowed error in total energy (the global break condition for the electronic SC-loop) is considered as 10-6 eV. The ground state geometries are optimized until the Hellman−Feynman atomic forces acting on each atom become less than 0.001 eV/Å.

The PDB file of Human telomeric DNA (PDB ID.- 1KF1) was retrieved from RCSB PDB, and the CIF files of telomeric DNA targeting small molecules (P5) were converted to PDB file using PyMOL (version 2.5.8). PDB files of both human telomeric DNA and telomeric DNA targeting small molecule (P5) fragment were then converted to PDBQT files using Graphical User Interface program AutoDock Tools (ADT). Polar hydrogens and united atom Kollman charges were assigned to the 1KF1 using ADT. Next a grid map on 1KF1 was prepared using grid box; the grid size was set to 110 × 92 × 112 xyz points with default grid spacing (i.e., 0.375 Å) and grid centre was designated at dimensions (x, y, and z): 25.206, -2.168 and -9.076; created a configuration WordPad file mentioning this information. Then AutoDock Vina was employed using this configuration file to dock the small molecule on human telomeric DNA.

The orientation that had the lowest binding energy or affinity was extracted and docked with the receptor molecule for further investigation.

UV-Vis Spectroscopy: UV-Vis absorption spectroscopy studies were carried out in Agilent spectrophotometer. Ligand solutions 10 μM C1, P1, P2, P3, P4, P5 and 5 μM C2 were titrated by gradual addition of 2 μL of preformed G4 DNA (10 μM). The titration was considered complete when there was no additional change observed in the maximum absorption values. The binding affinities were calculated following a reported protocol (J Med Chem. 2014 57(16):6973-88).

Fluorescence Spectroscopy: Fluorescence emission spectra were recorded on a Horiba Nano log spectrophotometer using quartz cells with a path length of 1 cm. 500 nM of the ligand, in 10 mM Tris−HCl (pH 7.4), 0.1 mM EDTA, 100 mM KCl. The solution of ligands and DNA were titrated against 10 μM preformed G4 DNA. Before recording each spectra samples were incubated for 5 mins. The titration was considered complete when there was no additional change observed in the fluorescence intensity values.

Confocal imaging: The A549 cells were seeded in duplicate at density of 3500 cells/well, in a 96-well plate, along with 20 μM compounds compound (C1, C2, P1, P2, P3, P4, P5, and 0.1 % DMSO) and incubated for 72 hours at 37 °C and 5% CO2 conditions in high glucose DMEM media, supplemented with 10% FBS, 1% Pen-Strep, and 0.1% Gentamycin. After 72 hours, cells were trypsinized using 0.25% 1X trypsin and seeded on glass coverslips along with compounds. After 16 hours, first the media were removed, then cells were fixed with 4% paraformaldehyde (PFA) by incubating this fixative with cells for 15 minutes at room temperature. After that, fixative was removed, and cells were washed twice with 1x PBS. Lastly, cells were incubated for 30 min with HCS NuclearMask Blue stain, protected from light, and then these coverslips were mounted on glass slides and imaged using a 60X oil objective on a confocal microscope under the DAPI channel.

FACS-Apoptosis: In a 24-well plate, A549 cells were seeded with 20 μM of each compounds (C1, C2, P1, P2, P3, P4, P5, and 0.1 % DMSO) in duplicate at 12000 cells/well in high glucose DMEM media, supplemented with 10% FBS, 1% Pen-Strep, and 0.1% Gentamycin. A549 cells were incubated for 72 hours at 37 °C and 5% CO2 conditions. After incubation, cells were trypsinized using 0.25% 1X trypsin and centrifuged at 2000 rpm for 2 min at 4°C, after that cells were washed once with 1X PBS. 200 μLof 1X Annexin V Binding buffer was added to resuspend the cell pellets, then 1 μL of Annexin V-FITC and 5 μL of 3 μM Propidium iodide is added to the cell suspension, and incubated on ice for 10 min. Cells were analysed using flow cytometry to study apoptosis.

Long term cytotoxicity: A549 cells were seeded (15000 cells/well) in a 24 well plate with high glucose DMEM media and incubated with 4 concentrations (20 μM, 10 μM, 5 μM, 2.5 μM) of each compound (C1, C2, P1, P2, P3, P4, P5, and 0.1 % DMSO). As the cells incubated with DMSO reached 90% confluency, cells were trypsinized using 0.25% 1X trypsin and 900 μL of high glucose DMEM media was added to each well. After resuspending, 800 μL of cell suspension from each well was transferred to separate centrifuge tubes. The remaining 200 μL

of cell suspension in each well was diluted in 1200 µL of media and from there 250 µL of cell suspension was transferred to each respective well of a new 24 well plate. The previously separated cell suspensions were centrifuged at 2000 rpm for 2 minutes to collect the cell pellets which were then incubated with 4% paraformaldehyde for 15 minutes at room temperature. After that, cells were again centrifuged down at the same speed and time and resuspended with PBS and stored at 4 ^{o}C. Cells in each tube were counted using flow cytometry where the no. of events were recorded for each sample at a fixed time.

Genotoxicity through γ-H2AX Detection:

A549 cells were seeded in a 24 well plate at a density of 15000 cells/ well, incubated for 7 days at 37 °C, 5% CO_2 condition in high glucose DMEM media, supplemented with 10% FBS, 1% Pen-Strep, and 0.1% Gentamycin with 20 µM, 10 µM of **P5** or 0.1% DMSO was taken as a control. After 7 days, cells were transferred to coverslips, and grown there for one day. Then cells were fixed with 4% paraformaldehyde (PFA) by incubating cells at room temperature for 15 min. Next, PFA was removed and washed thrice with 1X PBS, then 0.1% Triton-X 100 was added and incubated for 10 min at room temperature to permeabilize those cells. After that, cells were incubated with a blocking buffer (2% BSA dissolved in 1X PBS) for 1 hour at room temperature. Rabbit anti-gamma-H2AX primary antibody (1:5000 in 0.1% BSA) was added to the cells, incubated overnight at 4 °C followed by incubation in Alexa Fluor 594 goat anti-rabbit IgG (1:1000 in 0.1% BSA) along with HCS Nuclear Mask Blue stain at room temperature for 1 hour. Coverslips were then mounted on glass slides and imaged using a 60X oil objective on a confocal microscope under a 594/618 nm excitation/emission range and DAPI channel.

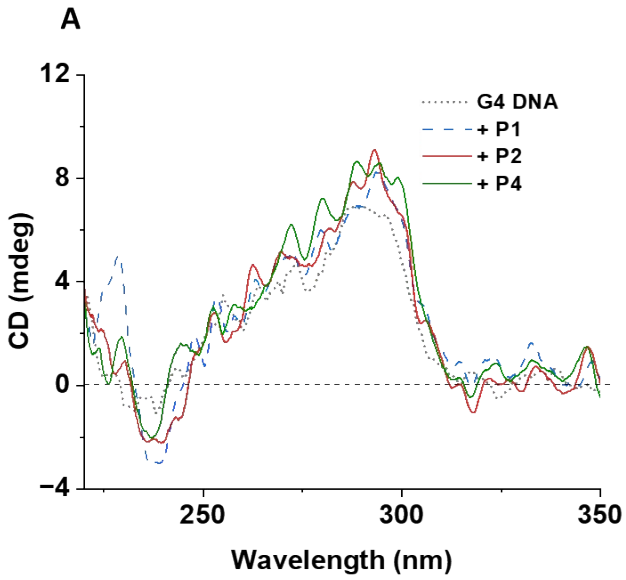


Fig S1. CD spectra of 3 µM Hum 21-mer G4 DNA alone, with compound C1 and P1, P2, and P4 (15 µM) in 10 mM Tris-HCl (pH 7.4) containing 0.1 M KCl, 0.1 mM EDTA

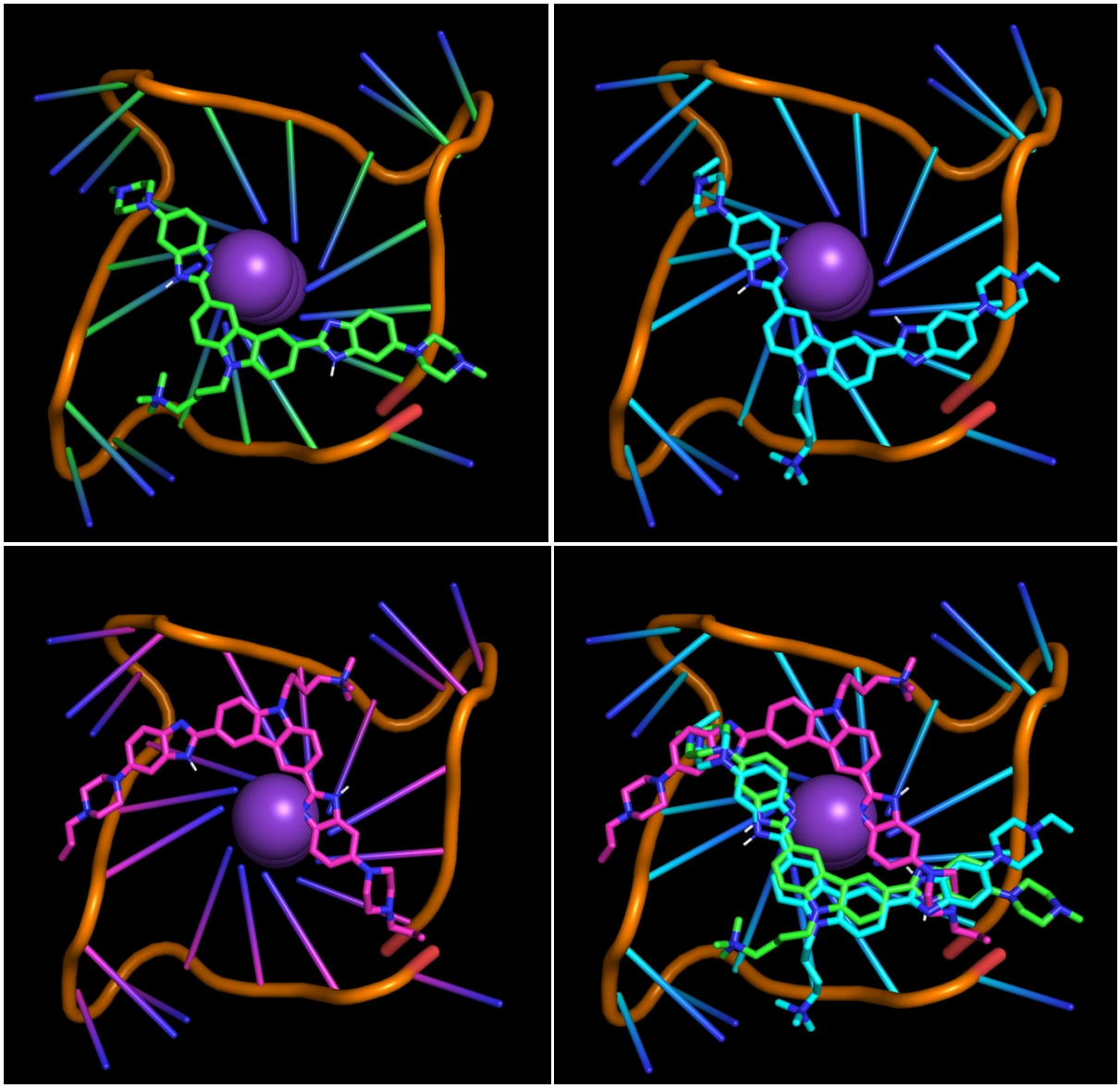


Fig S2. Molecular docking structure of C1a (top left), C1b (top right), C1c (bottom left) with 1KF1 and their superimposed (bottom right).

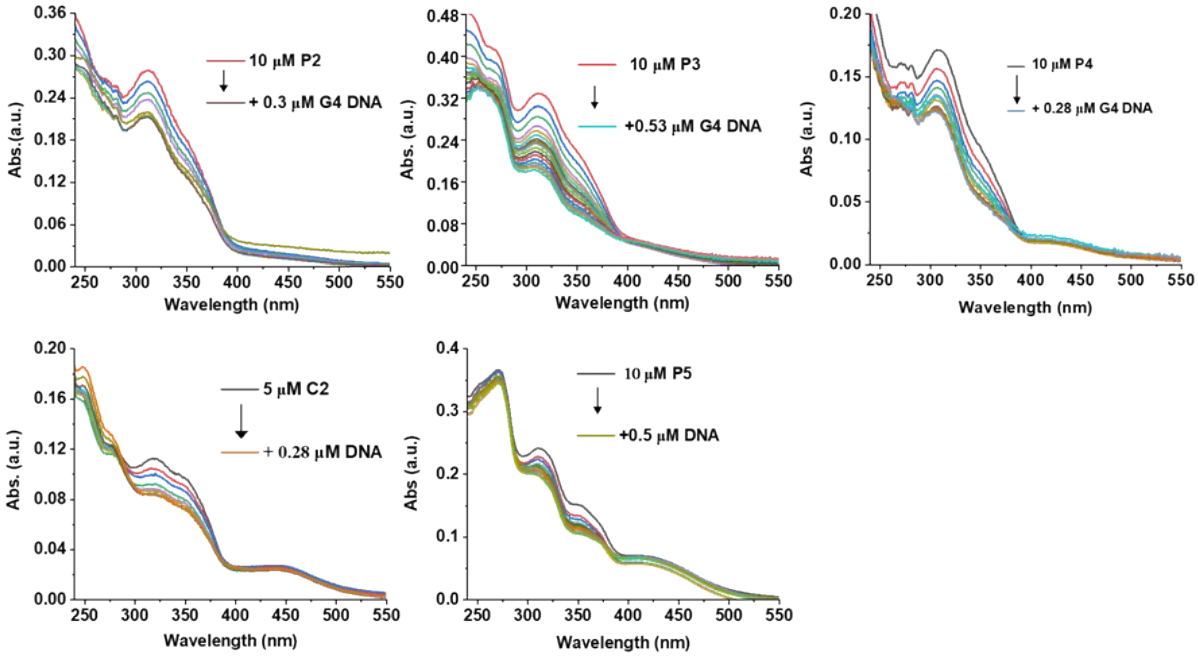
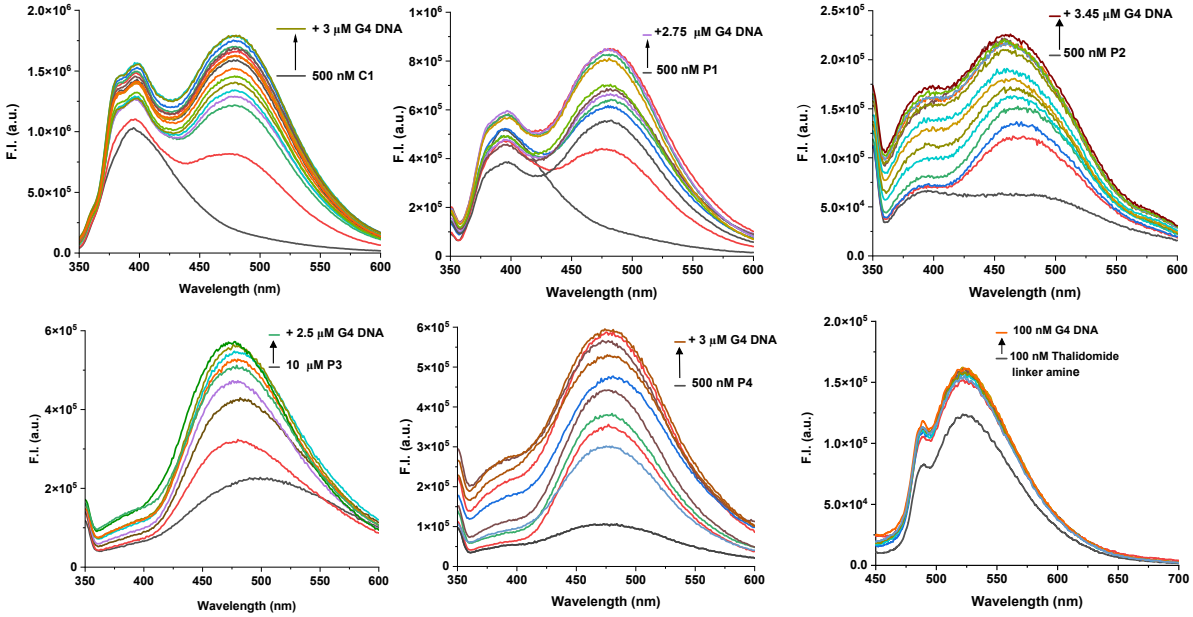


Fig S3. UV-Vis titration spectra of 10 μM ligand P2, P3, P4, C2 and P5 in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA and 0.1 M KCl with 10 μM pre-formed Hum 21-mer G4 DNA

Fig S4. Fluorescence titration spectra of 0.5 μM ligand C1, P1, P2, P3, and P4 and 0.1 μM

thalidomide detivative **23** respectively in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA and 0.1 M


+ 3 μM G4 DNA
500 nM C1
F.I. (a.u.)
Wavelength (nm)
+2.75 μM G4 DNA
500 nM P1
F.I. (a.u.)
Wavelength (nm)
+ 3.45 μM G4 DNA
500 nM P2
F.I. (a.u.)
Wavelength (nm)
+ 2.5 μM G4 DNA
10 μM P3
F.I. (a.u.)
Wavelength (nm)
+ 3 μM G4 DNA
500 nM P4
F.I. (a.u.)
Wavelength (nm)
100 nM G4 DNA
100 nM Thalidomide linker amine
F.I. (a.u.)
Wavelength (nm)

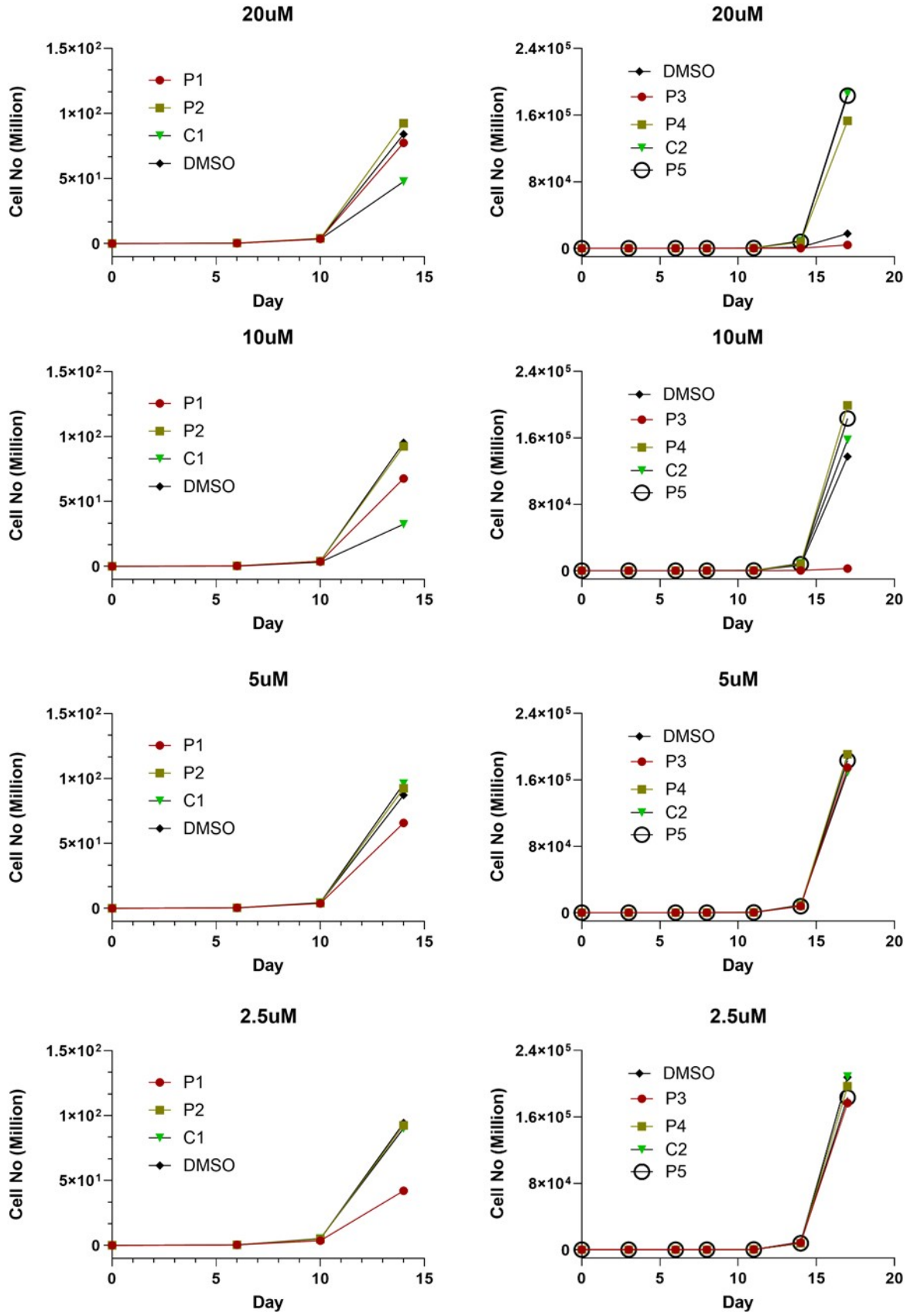


Fig S5. Effect of ligands on the cell viability upon long-term exposure (up to 17 days) to A549 as analysed by FACS.

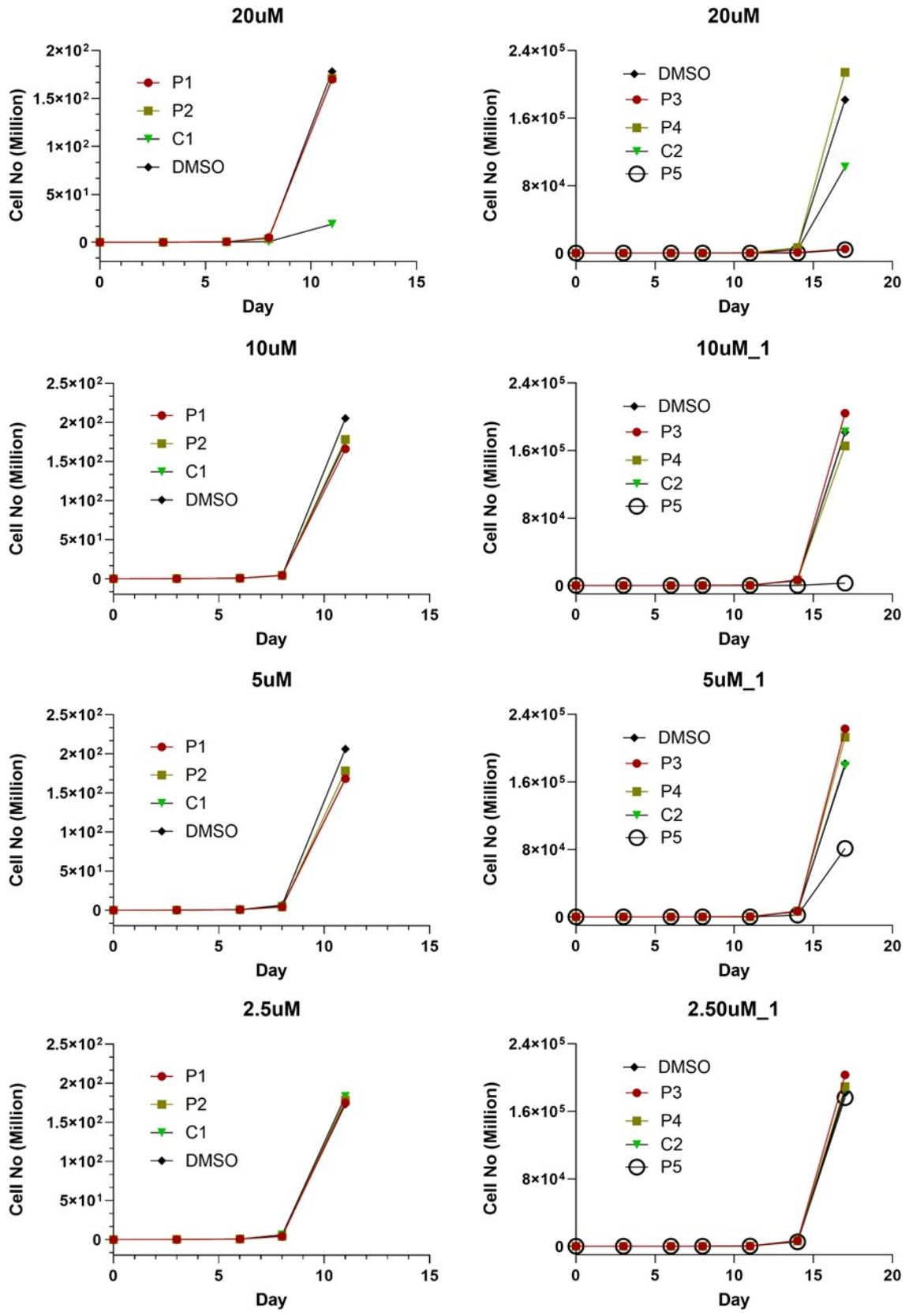


Fig S6. Effect of ligands on the cell viability upon long-term exposure (up to 17 days) to A549 as analysed by FACS.

References:

[1] X. Yang, Z. Wang, Y. Pei, N. Song, L. Xu, B. Feng, H. Wang, X. Luo, X. Hu, X. Qiu, H. Feng, Y. Yang, Y. Zhou, J. Li, B. Zhou, European Journal of Medicinal Chemistry 2021, 218, 113341.

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