# Supporting Information

# A High-Throughput Screen Reveals New Small-Molecule Activators and Inhibitors of Pantothenate Kinases

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## Contents

## 1. High-Throughput Screening (HTS)

The PanK3 HTS was performed using 520653 compounds from St. Jude compound library. For the detail of the HTS, including the PanK3 Kinase Glo assays, refer to our previously reported HTS of bioactive molecules.[1]

***Materials:***

DMSO, Tris-HCl, glycerol, MgCl2, pantothenate, ATP, γ-globulins and acetyl-CoA were obtained from Sigma. Staurosporine was purchased from LC Labs. HepG2 liver carcinoma cells and modified Eagle's minimal essential medium were obtained from the American Type Culture Collection (ATCC, Manassas, VA). L-glutamine, penicillin and streptomycin were obtained from Life Technologies. 2-(4-methoxyphenyl)benzo[d]thiazole was purchased from Chembridge. Kinase Glo® Plus and CellTiter Glo reagents were obtained from Promega.

***PanK1β dose response analysis***

The PanK3 Kinase Glo HTS protocol was followed with the exception that PanK3 protein was replaced with PanK1β protein. Because no potent PanK1β inhibitor was available, as a positive control for the inhibitor assay, PanK1β protein was omitted in the presence of ATP.

***Luciferase interference dose response analysis***

To evaluate the possible effect of compounds on interfering with the Kinase Glo assay system (and therefore might act as a false positive in a PanK-independent manner), the PanK3 Kinase Glo HTS protocol was followed with the exception that neither PanK3 nor PanK1β protein was added. 2-(4-methoxyphenyl)benzo[d]thiazole at 168 μM and DMSO were used as positive and negative control, respectively.[2]

***HepG2 cytotoxicity dose response analysis***

HepG2/C3A cells were maintained in modified Eagle's minimal essential medium with 10% FBS, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO2, and harvested and seeded into

white, solid-bottom, tissue culture-treated, 384-well polystyrene plates at a density of 2,500 cells per well in 25 µl media. Dilutions of various compounds (2.8 nM to 56 µM), staurosporine (56 µM, positive control), or DMSO (negative control) were transferred into individual wells. The assay plates were then incubated for three days at 37 °C, 5% CO2, 95% relative humidity followed by CellTiter Glo® (Promega) luminescence assay for cytotoxicity. Activity data were normalized to staurosporine (56 µM, as 100% inhibition) and 0.56% DMSO (as 0% Inhibition).

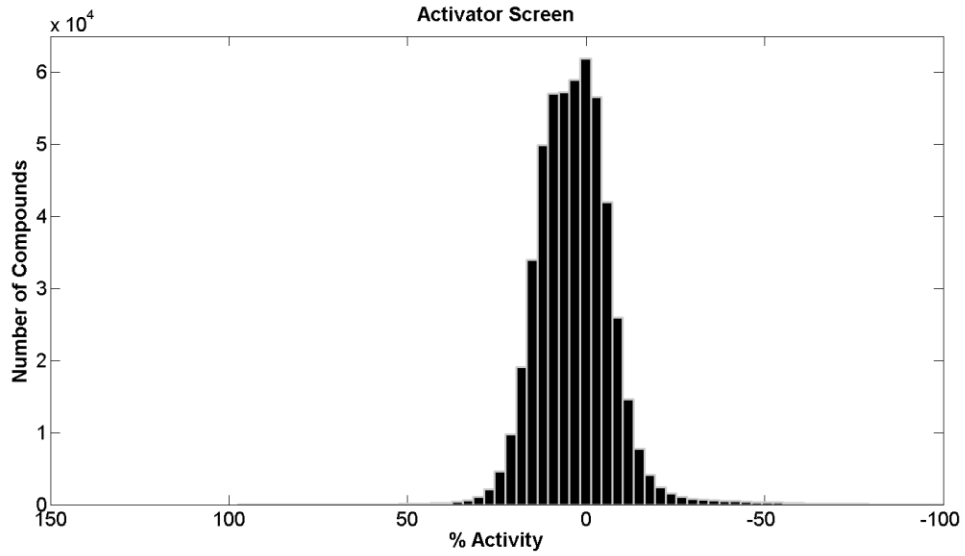



**Figure S1. Histogram for activator screen**

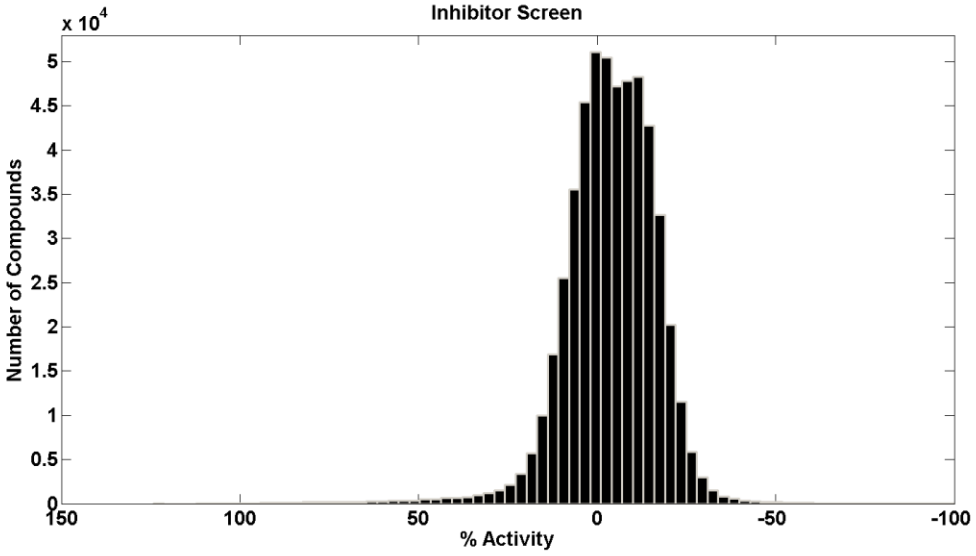



**Figure S2. Histogram for inhibitor screen**

## 2. ROC Analysis

Receiver operating characteristic (ROC) analysis was performed using custom R scripts (http://www.r-project.org/, v. 2.9.0) and the R rocr package (v. 1.0.2).

## 3. Biological assays

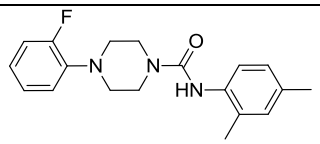
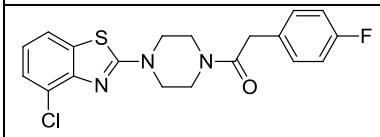
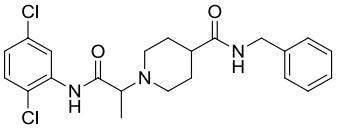
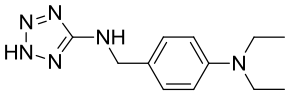
Human PanK isoforms were purified by affinity and gel filtration chromatography to homogeneity as described previously.[3]

**Radiochemical assay** was done as described previously.[1] Briefly 45 μM d-[1-$^{14}$C] pantothenate (specific activity 22.5 mCi/mmol), 2.5 mM ATP, 10 mM $MgCl_2$, 0.1 M Tris-HCl pH 7.5, 50 ng of protein in the presence of 0-1.2 μM compound 7 were incubated at 37°C for 10 minutes, then the reaction was stopped by the addition of 4 μl of 10% (v/v) acetic acid and was analyzed as described previously. To elucidate the mechanisms of PanK3 inhibition, the experiments were repeated in the presence of 0, 0.038 and 0.128 μM compound 7.

**Thermal shift assays** were done as described previously.[4] Briefly, 5 μM PanK3 was incubated with 2 mM ATP and 0-20 μM compound **7** in 20 mM HEPES (pH7.5), 10 mM $MgCl_2$ with 25X SYPRO Orange dye. Temperature was ramped from 25°C to 95°C in 70 cycles with 1°C increment per cycle in a Applied Biosystems 7500 Real Time thermal cycler. The fluorescence intensity was then plotted as a function of temperature to generate the two state transition curves. The Tm was calculated by the inflection point in the Boltzman equation curve.

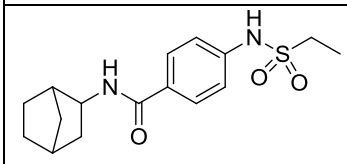
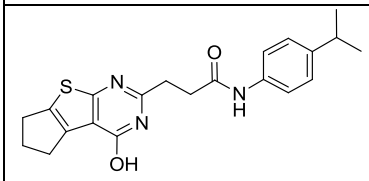
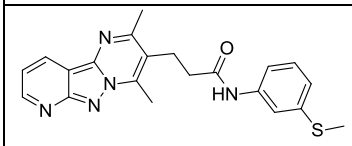
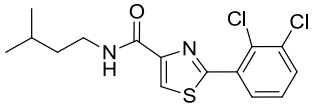
**Inhibition of CoA biosynthesis** by compound 7 in HepG2/C3A cells was done as described previously.[1] Briefly cells cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin, 1 mM octanoate were labeled with 1 μCi/mL [2,3-$^{3}$H]pantothenic acid (specific activity 50 Ci/mmol). Cells were incubated for 24 h and [$^{3}$H]CoA was quantified determining the amount of [$^{3}$H]CoA bound to the DEAE cellulose as described previously.

**Table S1.** Dose response analysis for activators

| Structure | ID | PanK-1 ACT $EC_{50}$ (μM)[a] | PanK-3 ACT $EC_{50}$ (μM)[a] | Luciferase $IC_{50}$ (μM) | HEP-G2 Cytox $IC_{50}$ (μM) |
|---|---|---|---|---|---|
|  | **1** | >56 | 2.41 ± 1.50 | >56 | >56 |
|  | **2** | >56 | 1.51 ± 1.35 | >56 | >56 |
|  | **3** | 3.55 ± 0.58 | 1.72 ± 0.39 | >56 | >56 |
|  | **4** | >56 | 3.43 ± 0.75 | >56 | >56 |

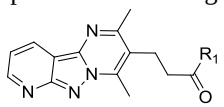
[a] The compounds were tested using ten-points concentration series in triplicate. The $IC_{50}$ values were calculated based on fitting the 30 data points to a single site binding model. Data are the average ± standard deviation.

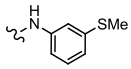
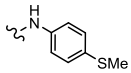
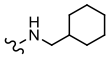
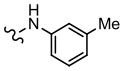
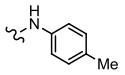
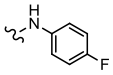
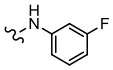
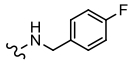
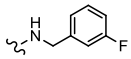
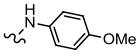
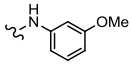
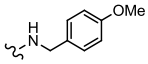
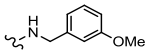
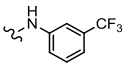
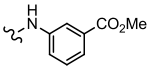
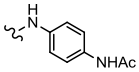
**Table S2.** Dose response analysis for inhibitors

| Structure | ID | PanK-1 Inh $IC_{50}$ (μM)[a] | PanK-3 Inh $IC_{50}$ (μM)[a] | Luciferase $IC_{50}$ (μM) | HEP-G2 Cytox $IC_{50}$ (μM) |
|---|---|---|---|---|---|
|  | **5** | 0.27 ± 0.05 | 0.18 ± 0.03 | >56 | >56 |
|  | **6** | 0.02 ± 0.02 | 0.12 ± 0.13 | >56 | >56 |
|  | **7** | 0.14 ± 0.02 | 0.36 ± 0.07 | >56 | >56 |
|  | **8** | 0.93 ± 0.63 | 2.45 ± 0.67 | >56 | >56 |

[a] The compounds were tested using ten-points concentration series in triplicate. The $IC_{50}$ values were calculated based on fitting the 30 data points to a single site binding model. Data are the average ± standard deviation.

**Table 1.** Side chain structure and inhibitory potencies of analogs of tricyclic compound **7**.



| ID | $R_1$ | Pank1β $IC_{50}$ (μM)[a] | Pank3 $IC_{50}$ (μM)[a] |
|---|---|---|---|
| **7** |  | 0.14 ± 0.02 | 0.36 ± 0.07 |
| **14** |  | 3.24 ± 1.29 | 10.51 ± 4.68 |
| **15** |  | 0.85 ± 0.19 | 24.50 ± 7.94 |
| **16** |  | >56 | >56 |
| **17** |  | 13.61 ± 3.37 | >56 |
| **18** |  | 1.22 ± 0.40 | 40.89 ± 4.48 |
| **19** |  | 1.57 ± 1.18 | 3.84 ± 1.55 |
| **20** |  | 11.16 ± 3.74 | 25.56 ± 15.94 |
| **21** |  | >56 | >56 |
| **22** |  | 0.24 ± 0.21 | 0.62 ± 0.24 |
| **23** |  | 0.55 ± 0.14 | 13.64 ± 2.88 |
| **24** |  | >56 | >56 |
| **25** |  | 15.78 ± 9.10 | 10.05 ± 3.14 |
| **26** |  | 6.69 ± 2.07 | 11.29 ± 5.56 |
| **27** |  | 0.28 ± 0.08 | 1.64 ± 0.69 |
| **28** |  | >56 | >56 |
| **29** |  | 14.69 ± 8.16 | 25.84 ± 10.82 |
| **30** |  | >56 | >56 |
| **31** |  | 0.19 ± 0.02 | 0.83 ± 0.41 |
| **32** |  | >56 | >56 |
| **33** |  | 0.64 ± 0.19 | 0.35 ± 0.19 |

[a] The compounds were tested using ten-points concentration series in triplicate. The $IC_{50}$ values were calculated based on fitting the 30 data points to a single site binding model. Data are the average ± standard deviation.

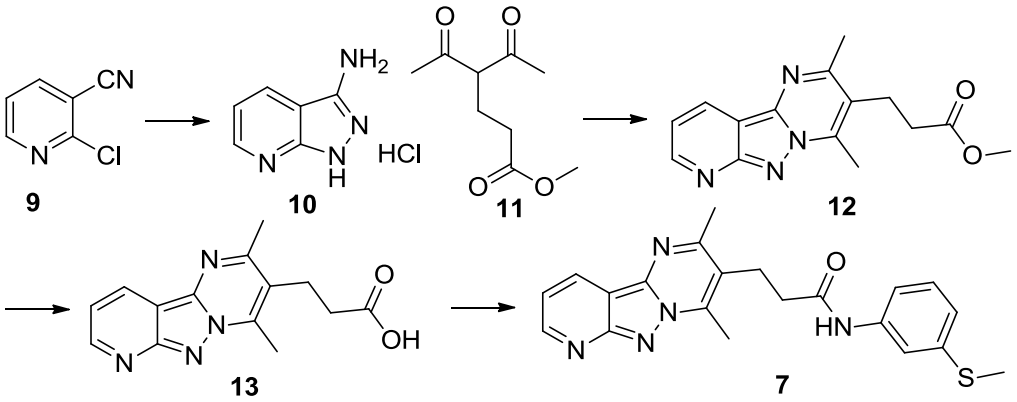
## 4. Chemistry

### 4.1. General Methods

Unless otherwise noted, all reactions were carried out in flame-dried glassware under a static nitrogen atmosphere with anhydrous solvent. All reagents were obtained from commercially available sources and used without purification. Purification was handled by reverse phase HPLC. All final compounds were purified to >95% purity indicated as averages of the total wave count (TWC) and the ELSD readings in LC/MS chromatogram (column: Acquity BEH C18). $^{1}H$ and $^{13}C$ spectra were recorded using 400 MHz, using DMSO as a solvent. The chemical shifts are reported in parts per million (ppm) relative to DMSO ($\delta$ 2.50 ppm for proton NMR and $\delta$ 39.50 ppm for carbon NMR). Coupling constants are reported in hertz (Hz). The following abbreviations are used to designate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. HRMS spectra acquired via Reverse-phase Acquity UPLC/XEVO G2 qTOF MS, Waters Instruments (Milford, MA).

### 4.2. Experimental Procedures

**Preparation of 3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(3-(methylthio)phenyl) propanamide (7)**.




**Synthesis of 10.** Chloropyrimidine (660 mg, 4.76 mmol) was dissolved in 15 mL of ethanol in a 30 mL microwave vial containing a stir bar and sealed. After purging with nitrogen, 0.746 mL of hydrazine (5 equivalents) was added and the vessel was microwaved for 30 minutes at 160 °C. Upon completion and cooling, the reaction was

concentrated *in vacuo* till dryness. The resulting solid was suspended in a 1:1:1 mixture of ethanol: ethyl acetate: hexanes, the remaining solid was filtered and washed with the same mixture described. High vacuum drying produced compound **10** in 2.56 g in an overall yield of 74% yield. Purity (>95%) was sufficient to continue on to the next step.

**Synthesis of 12.** To product **10** (2.56 g) dissolved in 13 mL of ethanol under nitrogen in a sealed 30 mL microwave vial containing a stir bar was added 4.1 mL of methyl 4-acetyl-5-oxohexanoate (1.5 equivalents). The mixture was microwaved at 80 °C for 15 minutes and deemed complete by HPLC, then concentrated *in vacuo* till dry. The crude product was concentrated a couple more times with ethyl acetate, then suspended in ethyl ether. Filtration of the solid and washing with more ethyl ether produced product **12** in 3.47 g, a 79% yield. Purity (>95%) was sufficient to proceed to the next reaction.

**Synthesis of 13.** Hydrolysis of **12** (3.05 g, 10.74 mmol) proceeded uneventfully in 55mL of THF and 18 mL of 1N sodium hydroxide for 2 hours. The resulting precipitate in the mixture was dissolved with water, washed a couple times with ethyl acetate, then concentrated *in vacuo* to afford a light beige solid (**13**, 2.9 g, 100% yield) that by HPLC was shown to be very clean product.

**Synthesis of 7.** To 2.0 g of **13** (7.404 mmol) dissolved in 20 mL of dry dimethylformamide (DMF) in a reaction vessel with stir bar was added 1.3 equivalents of N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) followed by 1.5 equivalents of triethylamine. After stirring for 5 minutes, 1.2 equivalents of 3-(methylthio)aniline was added and the mixture for stirred for 4 hours under nitrogen until deemed complete by HPLC. Direct reverse phase purification produced 1.2g of final product **7** as a white solid in 41% yield. **3-(2,4-dimethylpyrido-[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(3-(methylthio)phenyl) propanamide.** Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.00 (s, 1H), 8.83 (dd, *J* = 4.3, 1.8 Hz, 1H), 8.61 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.54 (t, *J* = 1.9 Hz, 1H), 7.31 (ddd, *J* = 8.2, 2.0, 1.1 Hz, 1H), 7.27 – 7.20 (m, 2H), 6.93 (ddd, *J* = 7.7, 1.9, 1.0 Hz, 1H), 3.23 – 3.16 (m, 2H), 2.95 (s, 3H), 2.76 (s, 3H), 2.69 – 2.61 (m, 2H), 2.44 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 170.03, 159.20, 156.63, 153.42, 143.48, 140.02, 139.49, 138.54, 130.08, 129.20, 123.57, 120.54, 116.09, 115.82, 115.56, 105.18, 35.75,

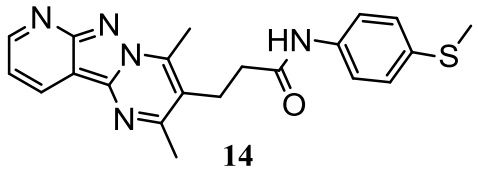
24.15, 23.00, 14.54, 14.06. HRMS: *m/z* calcd for C21H21N5OS+ [M+H]:392.1540; found 392.1539.

SMILE of compound 7:

CC1=NC2=C3C(N=CC=C3)=NN2C(C)=C1CCC(NC4=CC(SC)=CC=C4)=O.

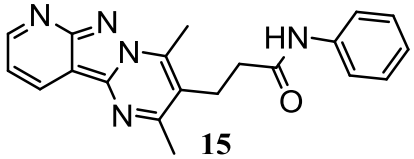
## Preparation and Characterization of selected library members:

Preparation of the following compounds was done in library format from 0.1 mmol of starting intermediate **13**, using the same protocol described above in the preparation of **7**.



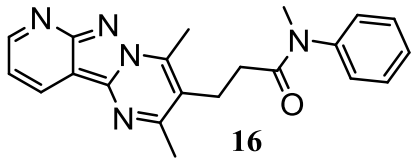
**14**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(4-(methylthio)-phenyl)propanamide (14)**. Avg. Purity (TWC & ELSD): 98.0%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.01 (s, 1H), 8.83 (m, 1H), 8.62 (m, 1H), 7.52 (m, 2H), 7.29 – 7.13 (m, 3H), 3.21 (m, 2H), 2.94 (s, 3H), 2.77 (s, 3H), 2.64 (m, 2H), 2.44 (s, 3H). HRMS: *m/z* calcd for C21H22N5OS+ [M+H]: 392.1545; found 392.1542.



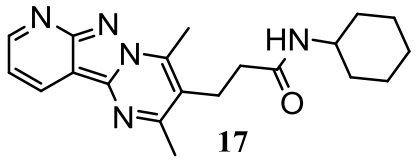
**15**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-phenylpropan-amide (15)**. Avg. Purity (TWC & ELSD): 98.3%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.02 (s, 1H), 8.91 – 8.78 (m, 1H), 8.71 – 8.56 (m, 1H), 7.57 (m, 2H), 7.30 (m, 3H), 7.12 – 6.97 (m, 1H), 3.30 – 3.15 (m, 2H), 2.98 (s, 3H), 2.80 (s, 3H), 2.74 – 2.60 (m, 2H). HRMS: *m/z* calcd for C20H20N5O+ [M+H]:346.1668; found 346.1663.



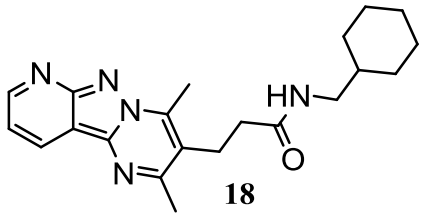
**16**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-methyl-N-phenyl-propanamide (16)**. Avg. Purity (TWC & ELSD): 99.8%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.82 (d, *J* = 6.8 Hz, 1H), 8.60 (t, *J* = 7.7 Hz, 1H), 8.25 (d, *J* = 6.9 Hz, 1H), 7.36 (m, 2H), 7.25 (m, 3H), 3.5 (obs. s, 3H), 3.18 (s, 3H), 3.08 (m, 2H), 2.74 (m, 3H), 2.53 (m, 3H), 2.42 – 2.26 (m, 2H). HRMS: *m/z* calcd for C21H22N5O+ [M+H]:360.1824; found 360.1823.



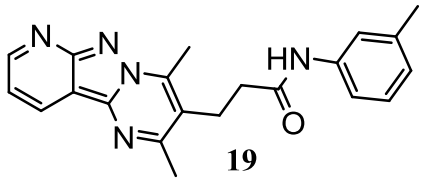
**17**

**N-cyclohexyl-3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)propan-amide (17)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.83 (dq, *J* = 5.2, 2.1 Hz, 1H), 8.62 (m, 1H), 7.76 (m, 1H), 7.25 (m, 1H), 3.46 (m, 1H), 3.16 – 3.03 (m, 2H), 2.93 (s, 3H), 2.74 (s, 3H), 2.45 – 2.31 (m, 2H), 1.71 – 1.43 (m, 4H), 1.20 (m, 2H), 1.02 (m, 2H). HRMS: *m/z* calcd for C20H26N5O+ [M+H]:352.2137; found 352.2138.



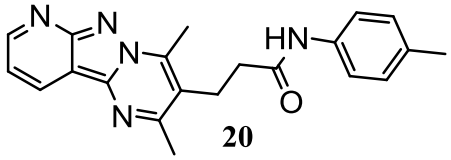
**18**

**N-(cyclohexylmethyl)-3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)propanamide (18)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.88 – 8.77 (m, 1H), 8.62 (t, *J* = 7.5 Hz, 1H), 7.87 – 7.74 (m, 1H), 7.24 (t, *J* = 6.4 Hz, 1H), 3.13 (t, *J* = 7.1 Hz, 2H), 2.93 (s, 3H), 2.79 (m, 5H), 2.56 – 2.36 (m, 6H), 1.38 (m, 6H), 1.18 (s, 1H), 0.95 (d, *J* = 10.9 Hz, 3H), 0.73 – 0.52 (m, 2H). HRMS: *m/z* calcd for C21H28N5O+ [M+H]:366.2294; found 366.2295.



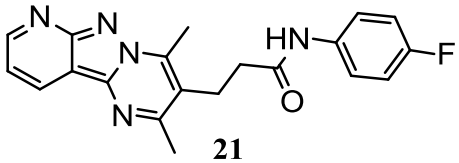
**19**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(m-tolyl)propanamide (19)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.92 (s, 1H), 8.83 (m, 1H), 8.61 (m, 1H), 7.37 (m, 2H), 7.21 (m, 2H), 6.85 (m, 1H), 3.20 (m, 2H), 2.95 (s, 3H), 2.77 (s, 3H), 2.65 (m, 2H), 2.25 (s, 3H). HRMS: *m/z* calcd for C21H28N5O+ [M+H]:360.1824; found 360.1827.



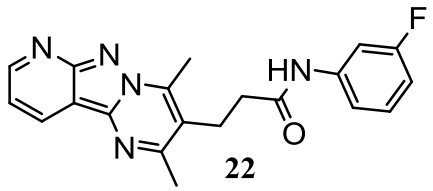
**20**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(p-tolyl)propanamide (20)**. Avg. Purity (TWC & ELSD): 99.7%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.92 (s, 1H), 8.83 (m, 1H), 8.61 (m, 1H), 7.44 (m, 2H), 7.25 (m, 1H), 7.09 (m, 2H), 3.20 (m, 2H), 2.95 (s, 3H), 2.77 (s, 3H), 2.64 (m, 2H), 2.24 (s, 3H) HRMS: *m/z* calcd for C21H28N5O+ [M+H]:360.1824; found 360.1827.



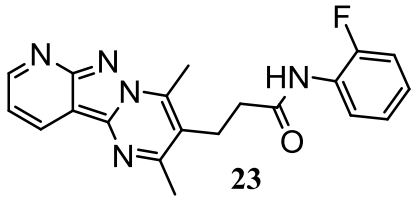
**21**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(4-fluorophenyl)-propanamide (21)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.16 (s, 1H), 8.92 – 8.78 (m, 1H), 8.73 – 8.56 (m, 1H), 7.69 – 7.50 (m, 2H), 7.37 – 7.05 (m, 3H), 3.30 – 3.13 (m, 2H), 2.97 (s, 3H), 2.79 (s, 3H), 2.53 (m, 2H). HRMS: *m/z* calcd for C20H19FN5O+ [M+H]:364.1573; found 364.1567.



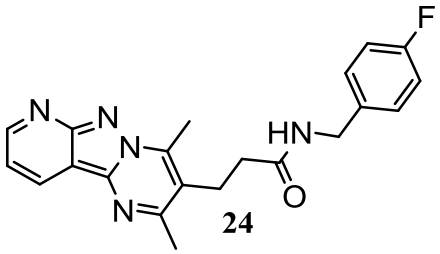
22

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(3-fluorophenyl)-propanamide (22)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.23 (s, 1H), 8.90 – 8.77 (m, 1H), 8.62 (m, 1H), 7.60 (m, 1H), 7.41 – 7.17 (m, 3H), 6.87 (m, 1H), 3.21 (m, 2H), 2.96 (s, 3H), 2.77 (s, 3H), 2.68 (m, 2H). HRMS: *m/z* calcd for C20H19FN5O+ [M+H]:364.1573; found 364.1571.



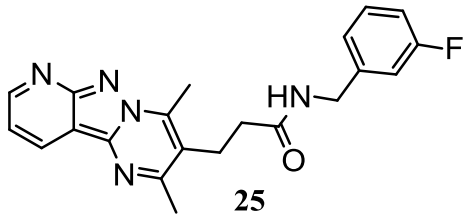
23

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(2-fluorophenyl)-propanamide (23)**. Avg. Purity (TWC & ELSD): 98.5%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.80 (s, 1H), 8.85 (ddd, *J* = 5.0, 3.1, 1.7 Hz, 1H), 8.64 (dq, *J* = 8.1, 1.8 Hz, 1H), 7.88 (d, *J* = 8.3 Hz, 1H), 7.31 – 7.19 (m, 1H), 7.15 (dq, *J* = 7.5, 2.4 Hz, 2H), 3.28 – 3.19 (m, 2H), 2.98 (d, *J* = 2.6 Hz, 3H), 2.83 – 2.71 (m, 5H). HRMS: *m/z* calcd for C20H19fN5O+ [M+H]:364.1495; found 364.1569.



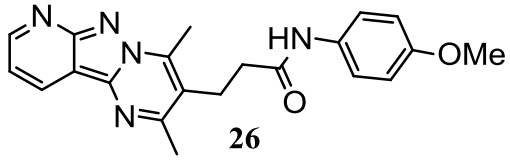
24

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(4-fluorobenzyl)-propanamide (24)**. Avg. Purity (TWC & ELSD): 99.7%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.98 – 8.78 (m, 1H), 8.77 – 8.55 (m, 1H), 8.55 – 8.34 (m, 1H), 7.40 – 6.85 (m, 4H), 4.36 – 4.11 (m, 2H), 3.28 – 3.06 (m, 2H), 3.05 – 2.85 (s, 3H), 2.85 – 2.65 (s, 3H). HRMS: *m/z* calcd for C21H21FN5O+ [M+H]:378.1730; found 378.1730.



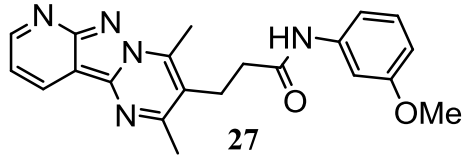
**25**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(3-fluorobenzyl)-propanamide (25)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.85 (m, 1H), 8.62 (m, 1H), 8.46 (s, 1H), 7.32 – 7.13 (m, 2H), 6.95 (m, 3H), 4.27 (m, 2H), 3.23 – 3.08 (m, 2H), 2.92 (s, 3H), 2.74 (s, 3H), 2.51 (m, 2H). HRMS: *m/z* calcd for C21H21FN5O+ [M+H]:378.173; found 378.1728.



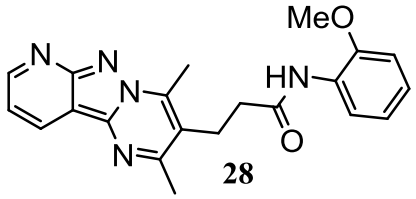
**26**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(4-methoxy-phenyl)propanamide (26)**. Avg. Purity (TWC & ELSD): 97.6%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.84 (s, 1H), 8.83 (m, 1H), 8.67 – 8.56 (m, 1H), 7.46 (m, 2H), 7.25 (m, 1H), 6.92 – 6.80 (m, 2H), 3.71 (s, 3H), 3.21 (m, 2H), 2.95 (s, 3H), 2.77 (s, 3H), 2.62 (m, 2H). HRMS: *m/z* calcd for C21H22N5O2+ [M+H]:376.1773; found 376.1770.



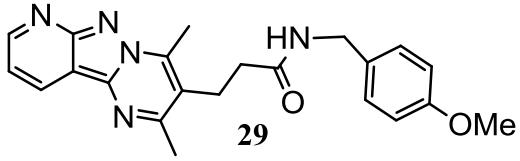
**27**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(3-methoxy-phenyl)propanamide (27)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.00 (s, 1H), 8.92 – 8.78 (m, 1H), 8.64 (m, 1H), 7.36 – 7.15 (m, 3H), 7.17 – 7.04 (m, 1H), 6.64 (m, 1H), 3.73 (s, 3H), 3.29 – 3.16 (m, 2H), 2.98 (s, 3H), 2.79 (s, 3H), 2.67 (m, 2H). HRMS: *m/z* calcd for C21H22N5O2+ [M+H]:376.1773; found 376.1772.



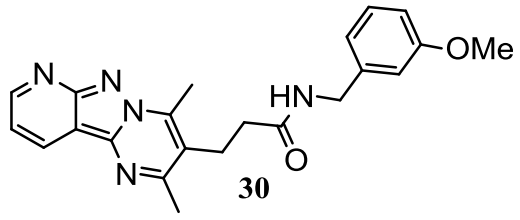

**28**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(2-methoxy-phenyl)propanamide (28)**. Avg. Purity (TWC & ELSD): 94.9%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.21 (s, 1H), 8.83 (m, 1H), 8.69 – 8.54 (m, 1H), 7.91 (m, 1H), 7.31 – 7.18 (m, 1H), 7.13 – 6.82 (m, 3H), 3.67 (s, 3H), 3.26 – 3.12 (m, 2H), 2.95 (s, 3H), 2.76 (s, 3H), 2.52 (m, 2H). HRMS: *m/z* calcd for C21H22N5O2+ [M+H]:376.1773; found 376.1772.



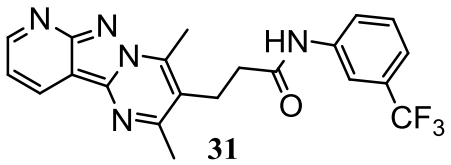

**29**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(4-methoxy-benzyl)propanamide (29)**. Avg. Purity (TWC & ELSD): 99.6%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.97 – 8.78 (m, 1H), 8.75 – 8.57 (m, 1H), 8.42 – 8.23 (m, 1H), 7.38 – 7.19 (m, 1H), 7.03 – 6.84 (m, 2H), 6.76 – 6.54 (m, 2H), 4.26 – 4.07 (m, 2H), 3.69 – 3.52 (s, 3H), 3.2 (m, 2H), 2.96 (s, 3H), 2.72 (s, 3H), 2.62 – 2.41 (m, 2H). HRMS: *m/z* calcd for C22H24N5O2+ [M+H]:390.1930; found 390.1932.



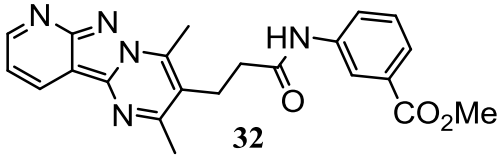

**30**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(3-methoxy-benzyl)propanamide (30)**. Avg. Purity (TWC & ELSD): 98.0%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.93 – 8.79 (m, 1H), 8.63 (m, 1H), 8.39 (m, 1H), 7.26 (m, 1H), 7.06 (m, 1H), 6.67 (m, 3H), 4.22 (m, 2H), 3.65 (s, 3H), 3.16 (m, 2H), 2.94 (s, 3H), 2.76 (s, 3H), 2.52 (m, 2H). HRMS: *m/z* calcd for C22H24N5O2+ [M+H]:390.1930; found 390.1932.



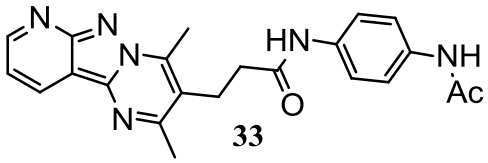
**31**

**3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)-N-(3-(trifluoro-methyl)phenyl)propanamide (31)**. Avg. Purity (TWC & ELSD): 100%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.34 (s, 1H), 8.84 (dd, *J* = 4.3, 1.8 Hz, 1H), 8.64 (dd, *J* = 8.1, 1.7 Hz, 1H), 8.08 (d, *J* = 2.0 Hz, 1H), 7.83 – 7.72 (m, 1H), 7.55 (t, *J* = 8.0 Hz, 1H), 7.46 – 7.36 (m, 1H), 7.26 (dd, *J* = 8.1, 4.3 Hz, 1H), 3.24 (dd, *J* = 9.0, 6.8 Hz, 2H), 2.98 (s, 3H), 2.79 (s, 3H), 2.74 – 2.65 (m, 2H). HRMS: *m/z* calcd for C21H19f3N5O+ [M+H]:414.1541; found 414.1547.



**32**

**Methyl-3-(3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)propan-amido)benzoate (32)**. Avg. Purity (TWC & ELSD): 89.2%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.23 (s, 1H), 8.83 (s, 1H), 8.62 (m, 1H), 8.23 (s, 1H), 7.84 (s, 1H), 7.63 (s, 1H), 7.46 (m, 1H), 7.25 (s, 1H), 3.84 (s, 3H), 3.23 (m, 2H), 2.96 (s, 3H), 2.77 (s, 3H), 2.51 (m, 2H). HRMS: *m/z* calcd for C22H22N5O3+ [M+H]:404.1722; found 404.1724.

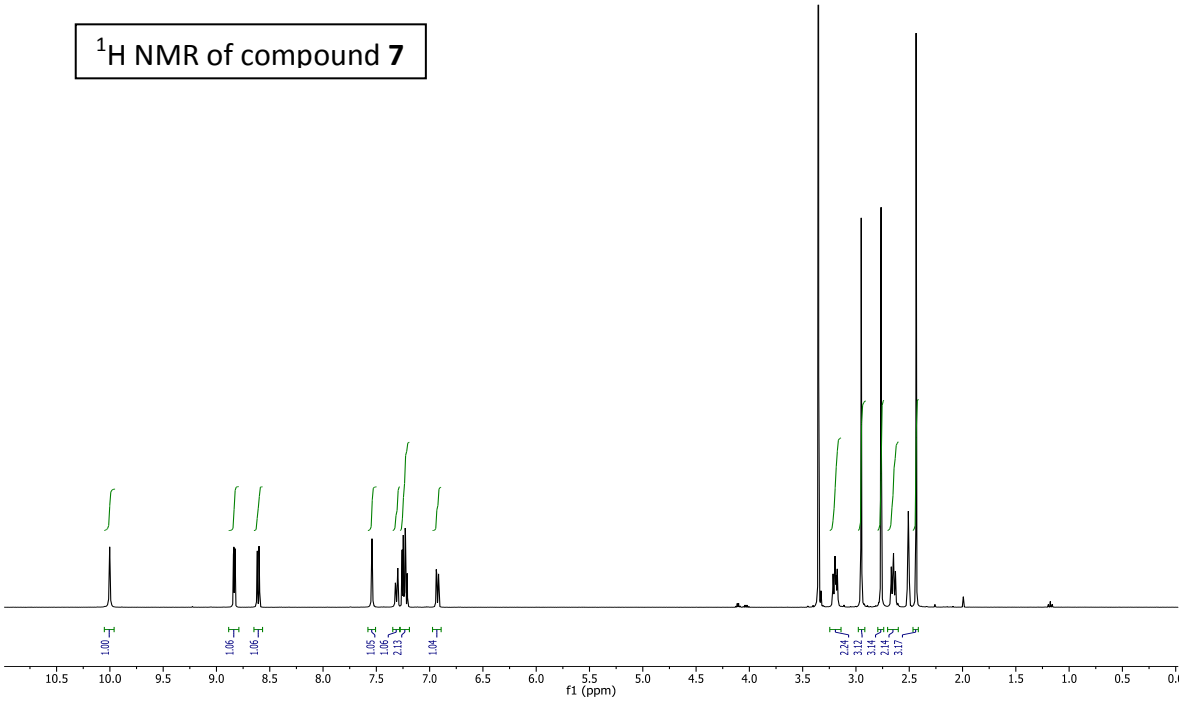


**33**

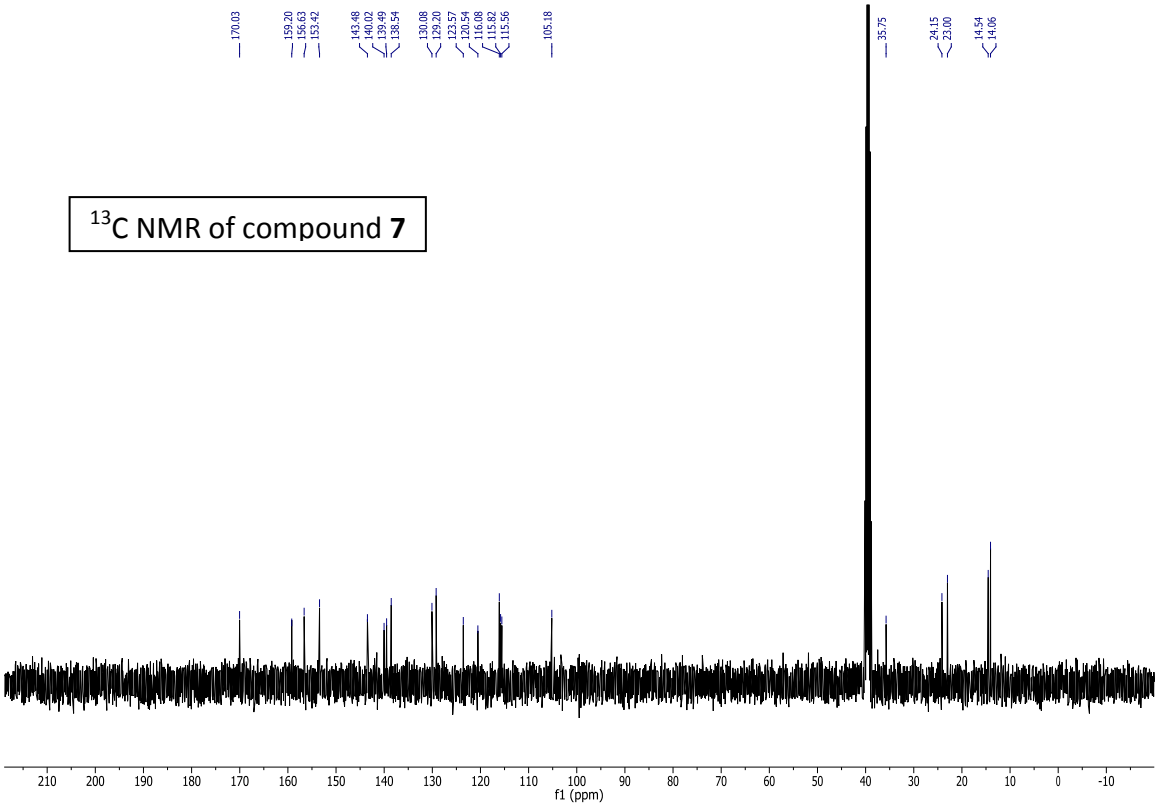
**N-(4-acetamidophenyl)-3-(2,4-dimethylpyrido[2',3':3,4]pyrazolo[1,5-a]pyrimidin-3-yl)propanamide (33)**. Avg. Purity (TWC & ELSD): 97%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.90 (s, 1H), 8.83 (m, 1H), 8.62 (m, 1H), 7.47 (m, 2H), 7.25 (m, 2H), 3.19 (m, 2H), 2.94 (s, 3H), 2.76 (s, 3H), 2.62 (m, 2H), 2.02 (s, 3H). HRMS: *m/z* calcd for C22H23N6O2+ [M+H]:403.1882; found 403.1880.

## 4.3. Copies of NMR of compound 7

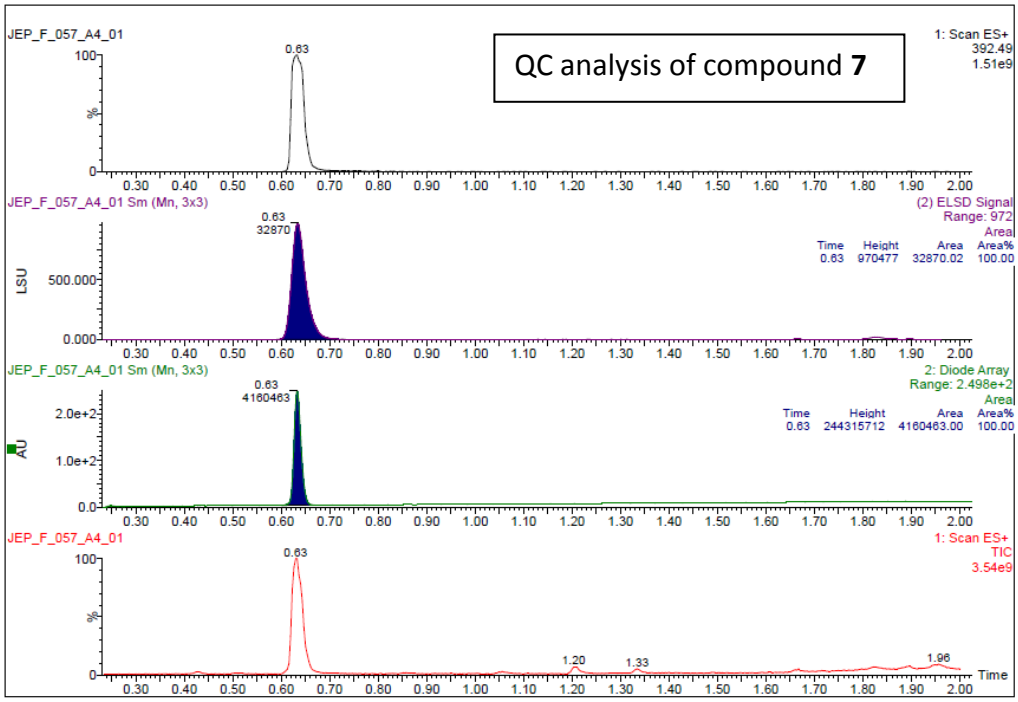
$^{1}$H NMR of compound **7**

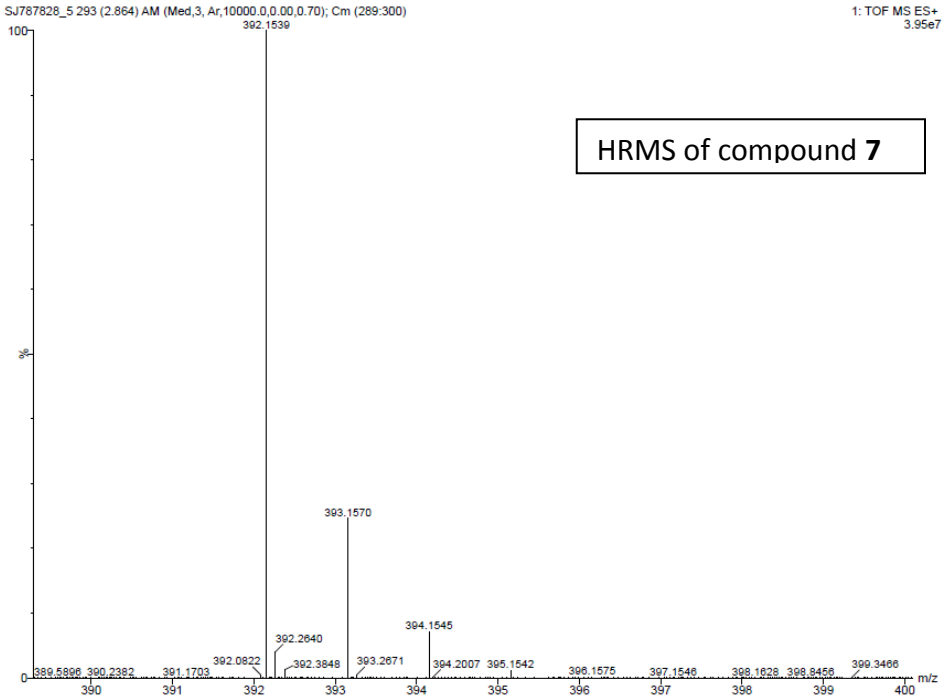


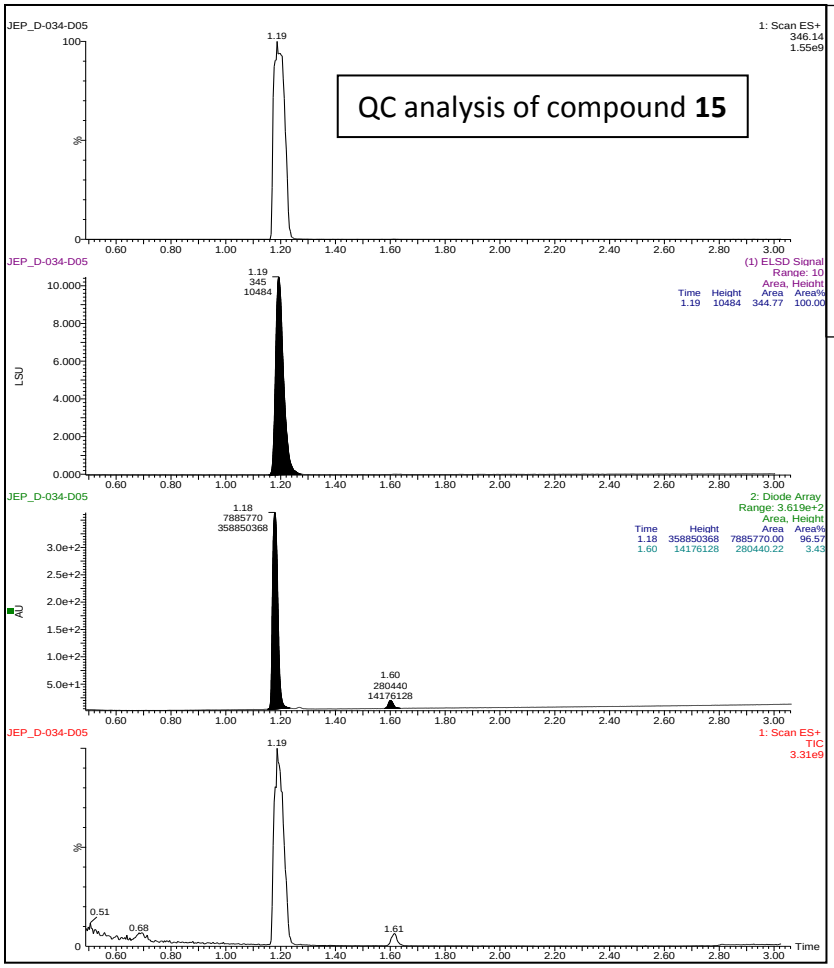

$^{13}$C NMR of compound **7**



## 4.4.Copies of QC analysis and mass spectra

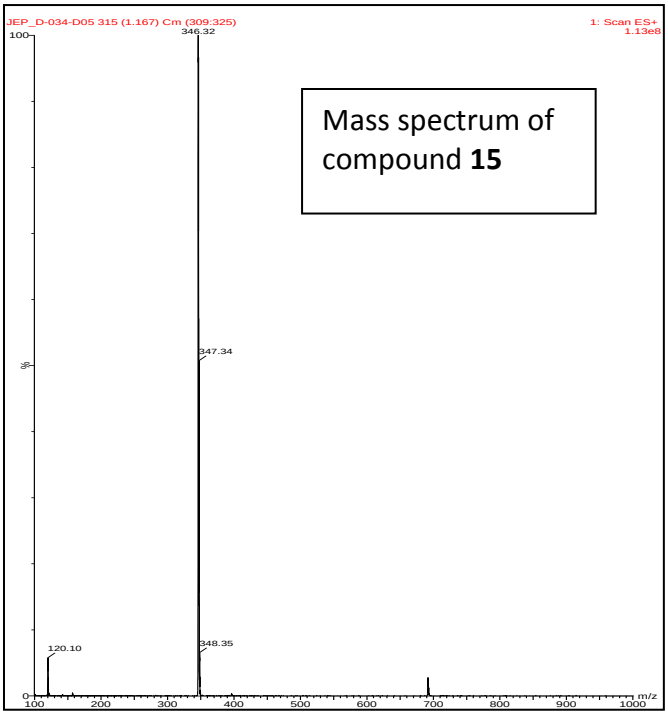


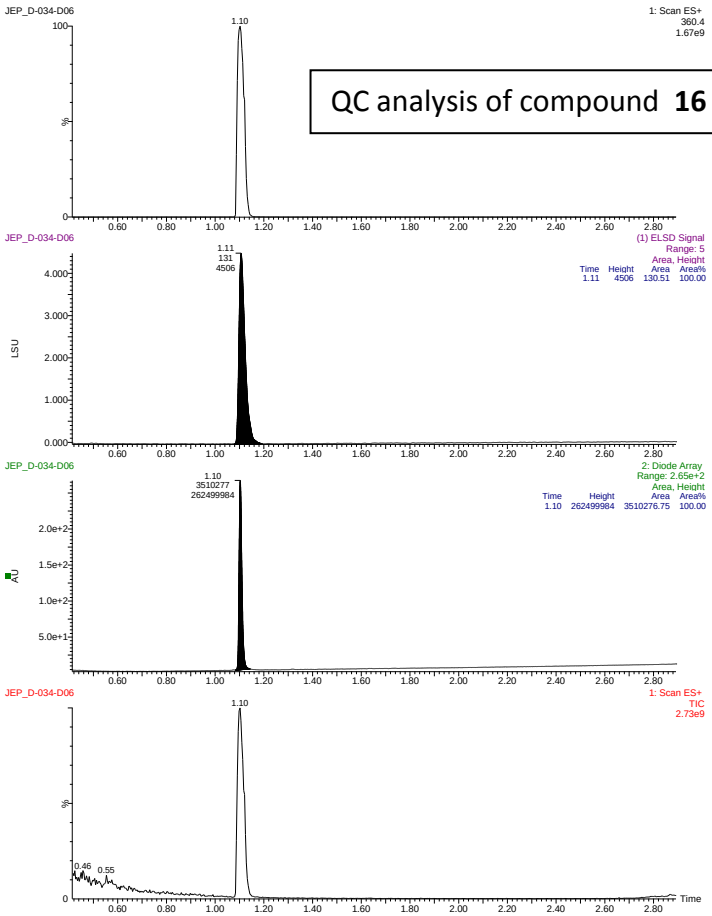






**Method Conditions**
Mobile Phase A: Water/0.1%formic acid
Mobile Phase B: Acetonitrile/0.1% formic acid
**Column**: Acquity BEH C18
2.1 x 50 mm, 1.7 um
**Gradient**:

| | Time | Flow Rate | %A | %B |
|---|---|---|---|---|
| 1. | I | 0.600 | 90.0 | 10.0 |
| 2. | 2.50 | 0.600 | 5.0 | 95.0 |
| 3. | 2.90 | 0.600 | 5.0 | 95.0 |
| 4. | 2.95 | 0.600 | 90.0 | 10.0 |

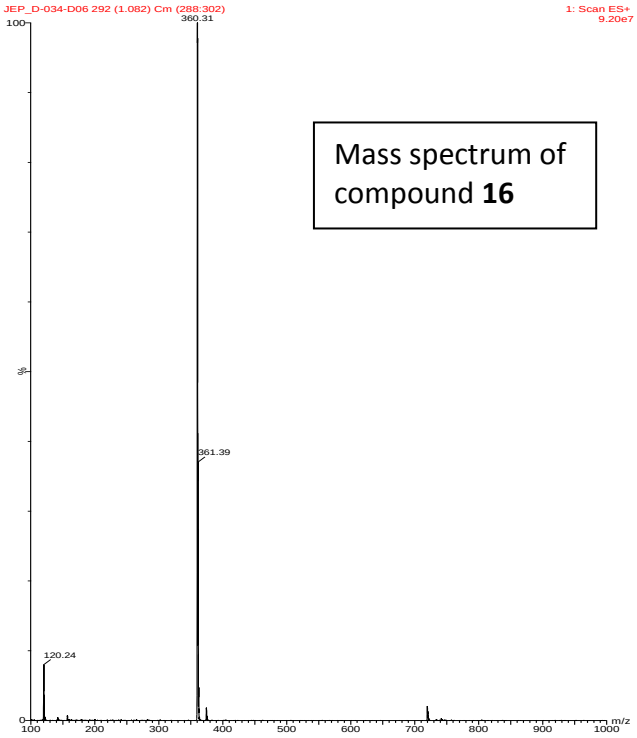




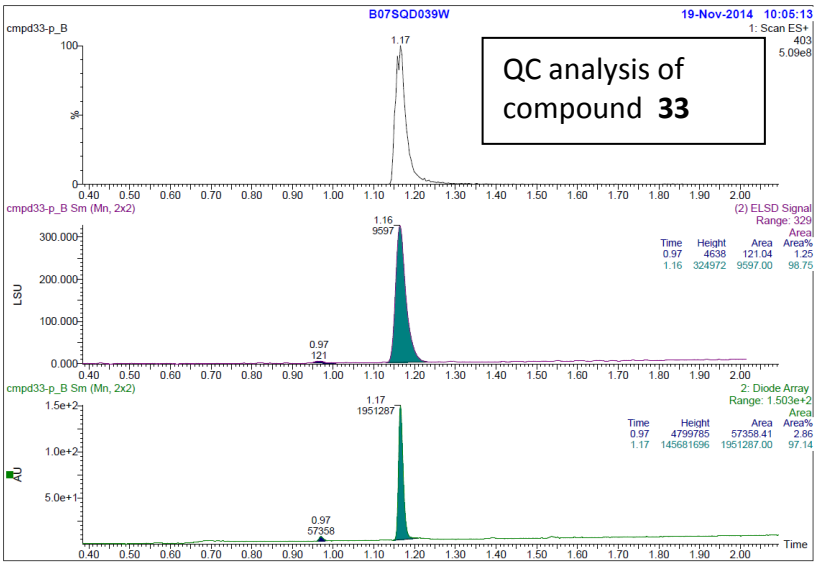

**Method Conditions**
Mobile Phase A: Water/0.1%formic acid
Mobile Phase B: Acetonitrile/0.1% formic acid
**Column**: Acquity BEH C18
2.1 x 50 mm, 1.7 um
**Gradient**:

| Time | Flow Rate | %A | %B |
|---|---|---|---|
| 1. I | 0.600 | 90.0 | 10.0 |
| 2. 2.50 | 0.600 | 5.0 | 95.0 |
| 3. 2.90 | 0.600 | 5.0 | 95.0 |
| 4. 2.95 | 0.600 | 90.0 | 10.0 |

QC analysis of compound **16**

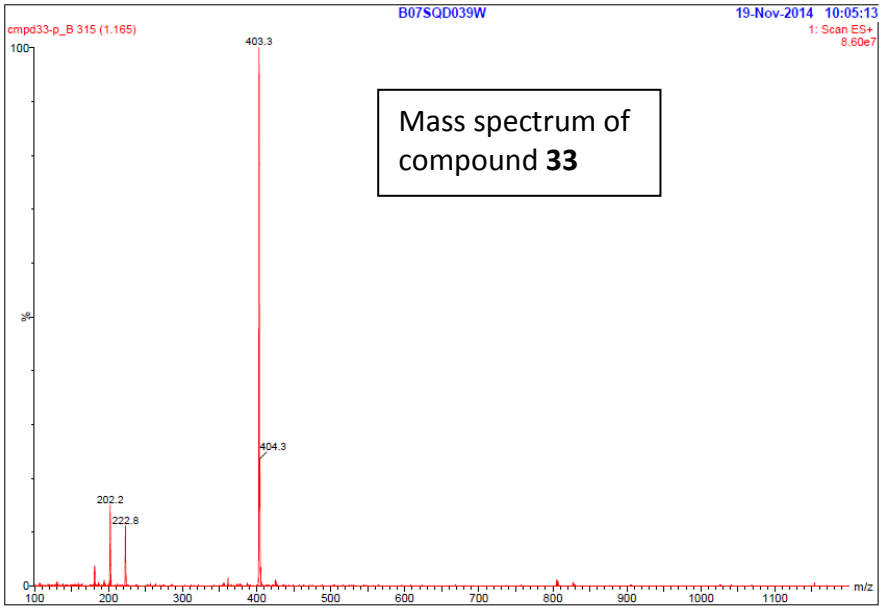



Mass spectrum of compound **16**




**Method Conditions**
Mobile Phase A: Water/0.1%formic acid
Mobile Phase B: Acetonitrile/0.1% formic acid
**Column**: Acquity BEH C18
2.1 x 50 mm, 1.7 um
**Gradient**:

| Time | Flow Rate | %A | %B |
|---|---|---|---|
| 1. I | 0.600 | 98.0 | 2.0 |
| 2. 0.2 | 0.600 | 98.0 | 2.0 |
| 3. 2.60 | 0.600 | 5.0 | 95.0 |
| 4. 2.90 | 0.600 | 5.0 | 95.0 |




## 5. References for supporting information

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