Electronic Supplementary Information (ESI)

Triaminopyrimidine derivatives as transmembrane HCl transporters

Petr Motloch, [a] Ana Guerreiro, [b] Carolina Q. Azeredo, [b] Gonçalo J. L. Bernardes,[a,b] Christopher A. Hunter [a] and Istvan Kocsis *[a]

[a]*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, U.K.*

[b]*Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal.*

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1. General information

The chemicals were bought from commercial suppliers and used without further purifications. For chromatography, automatic chromatography systems CombiFlash® R_f^+ and CombiFlash® R_f^+ Lumen™ (with UV light detection: 254 nm and 280 nm) with pre-packed puriFlash® columns from Interchim (silica, 25 µm) with a loading of mixtures on Celite®. The microwave used was Biotage Initiator$^+$. The reactions were monitored by LCMS Waters Acquity H-class UPLC coupled with a single quadrupole Waters SQD2 with the conditions as follows: UPLC Column ACQUITY UPLC HSS T3, 100Å, 1.8 µm, 2.1 mm X 50 mm; solvent A: Water + 0.1% formic acid; solvent B: acetonitrile + 0.1% formic acid; gradient and flow rate (see below); column temperature of 40 °C.

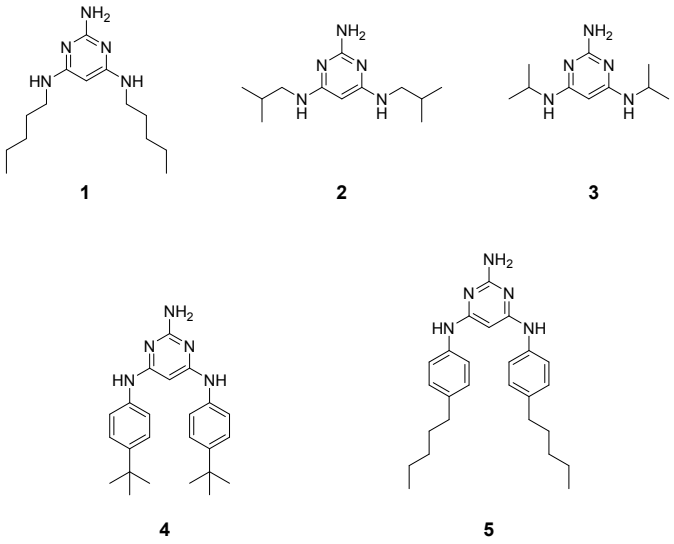
Methods

FAST: Gradient: 0 – 2 minutes 5% – 100%B + 1 minute 100% B
Flow rate: 0.6 ml/min

SLOW: Gradient: 0 – 4 minutes 5% – 100%B + 1 minute 100% B
Flow rate: 0.6 ml/min

^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz Avance III HD Spectrometer at 400 MHz for ^{1}H and 101 MHz for ^{13}C. All chemicals shift are quoted in ppm and were referenced to the residual peaks of $CDCl_3$ (^{1}H: 7.26 ppm; ^{13}C: 77.00 ppm), CD_3OD (^{1}H: 3.31 ppm; ^{13}C: 49.00 ppm) or d^6-DMSO (^{1}H: 2.50 ppm). Coupling constants *J* are stated in Hz. FT-IR spectra were measured on a Bruker Alpha spectrometer. HR-MS spectra were obtained on a Waters Xevo® G2-S or a Waters LCT Premier by electrospray-ionization of samples. Melting points were recorder on a Mettler-Toledo MP90 system.

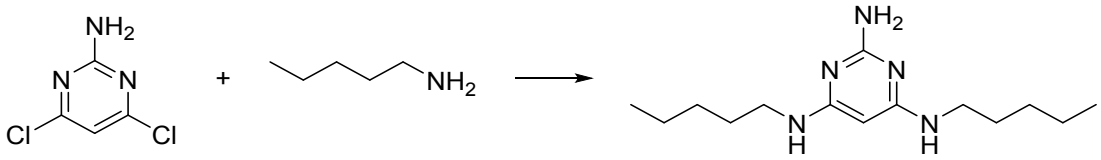
2. Molecular structures of compounds 1-5



ESI-Figure 1. Molecular structure of studied compounds

3. Synthesis and characterization

N4,N6-dipentylpyrimidine-2,4,6-triamine (1)



4,6-Dichloropyrimidin-2-amine (210 mg, 1.28 mmol) was flushed with nitrogen in MW vial. Amylamine (2.5 mL, 21.4 mmol) was added and the mixture was heated in microwave at 130 °C for 14 hours. The remaining amylamine was removed under reduced pressure and the residue was loaded on Celite. A combiflash of the residue on silica (EtOAc/MeOH: MeOH 0%→5%→10%) provided the title compound (210 mg, 62% yield) as a slightly-pale-brown solid.

^{1}H NMR ($CDCl_3$, 400 MHz, 298 K): δ 4.81 (s, 1H), 4.45 – 4.41 (m, 4H), 3.14 (dd, J = 13.0, 7.0 Hz, 4H), 1.62 – 1.54 (m, 4H), 1.40 – 1.30 (m, 8H), 0.92 – 0.89 (m, 6H).
^{13}C NMR ($CDCl_3$, 101 MHz, 298 K): δ 164.3, 162.3, 71.9, 41.7, 29.1, 29.1, 22.4, 14.0.

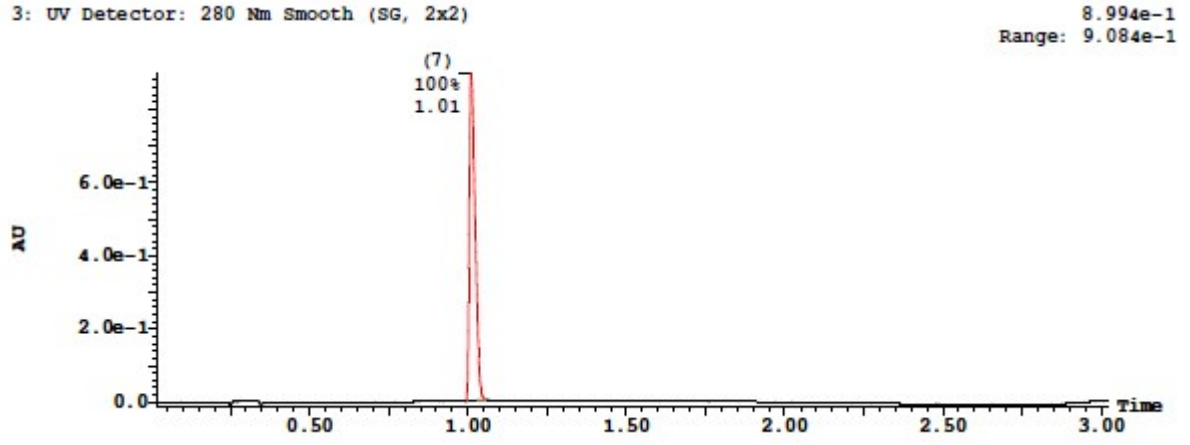
HR-MS (ESI): Calculated for $C_{14}H_{28}N_5$ $[M+H]^+$ 266.2345, found: 266.2348 (Δ = 1.1 ppm)

FT-IR (thin film): 3310, 2955, 2928, 2858, 1580, 1433, 1368 cm^{-1}.

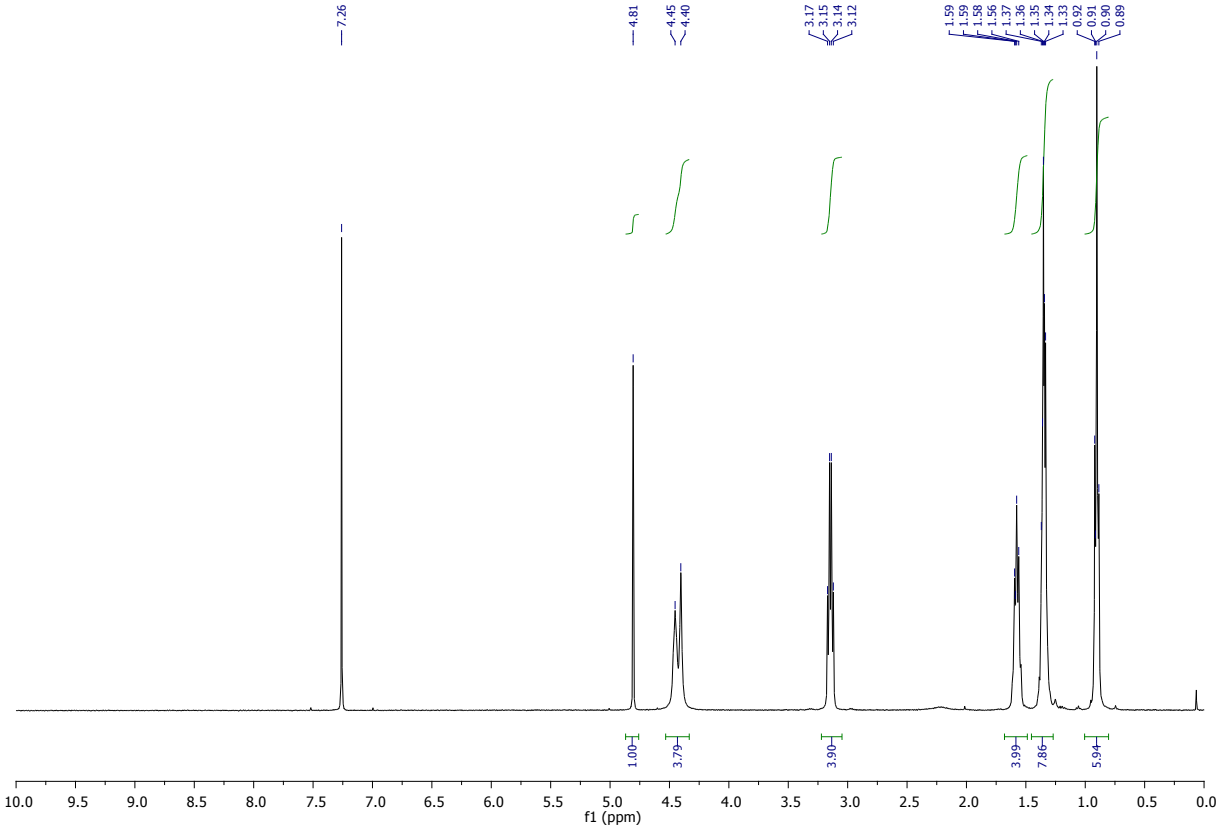
MP: 85 – 87 °C

LCMS Method: FAST

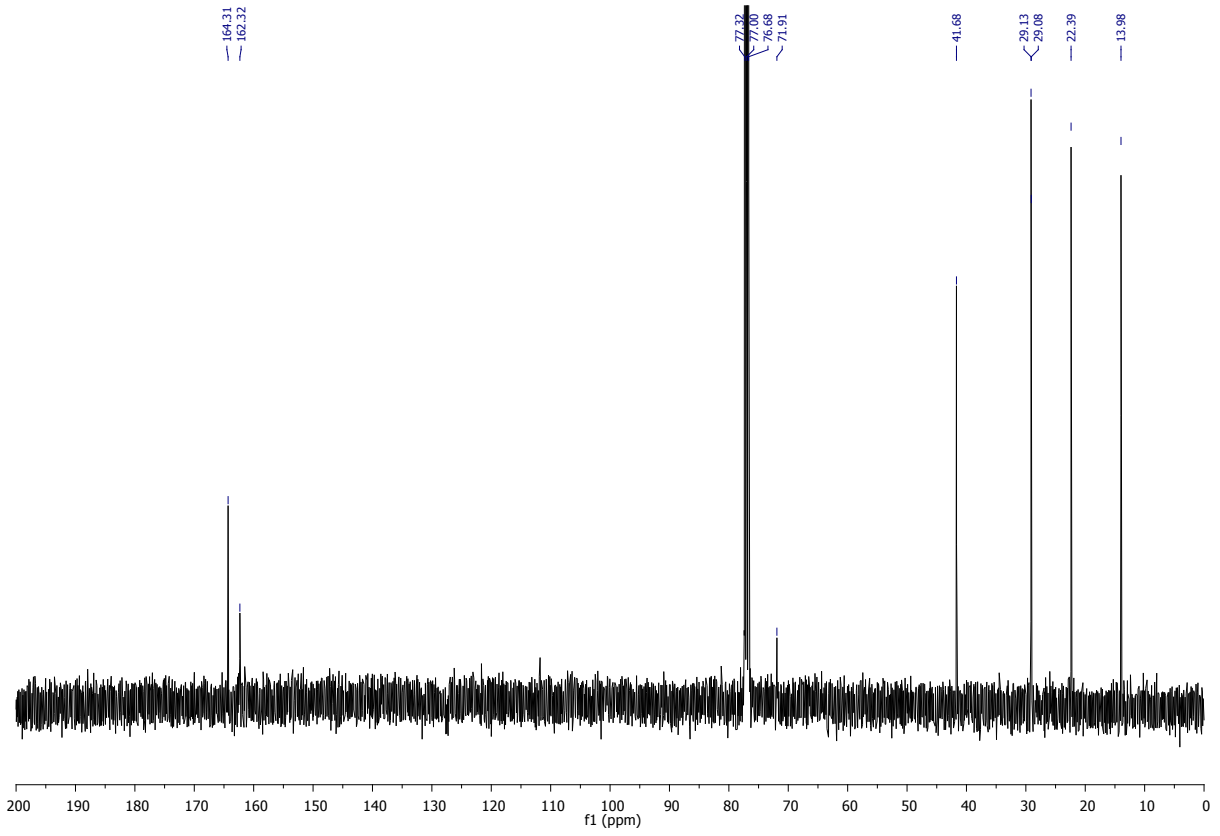
LCMS



^{1}H NMR



^{13}C NMR



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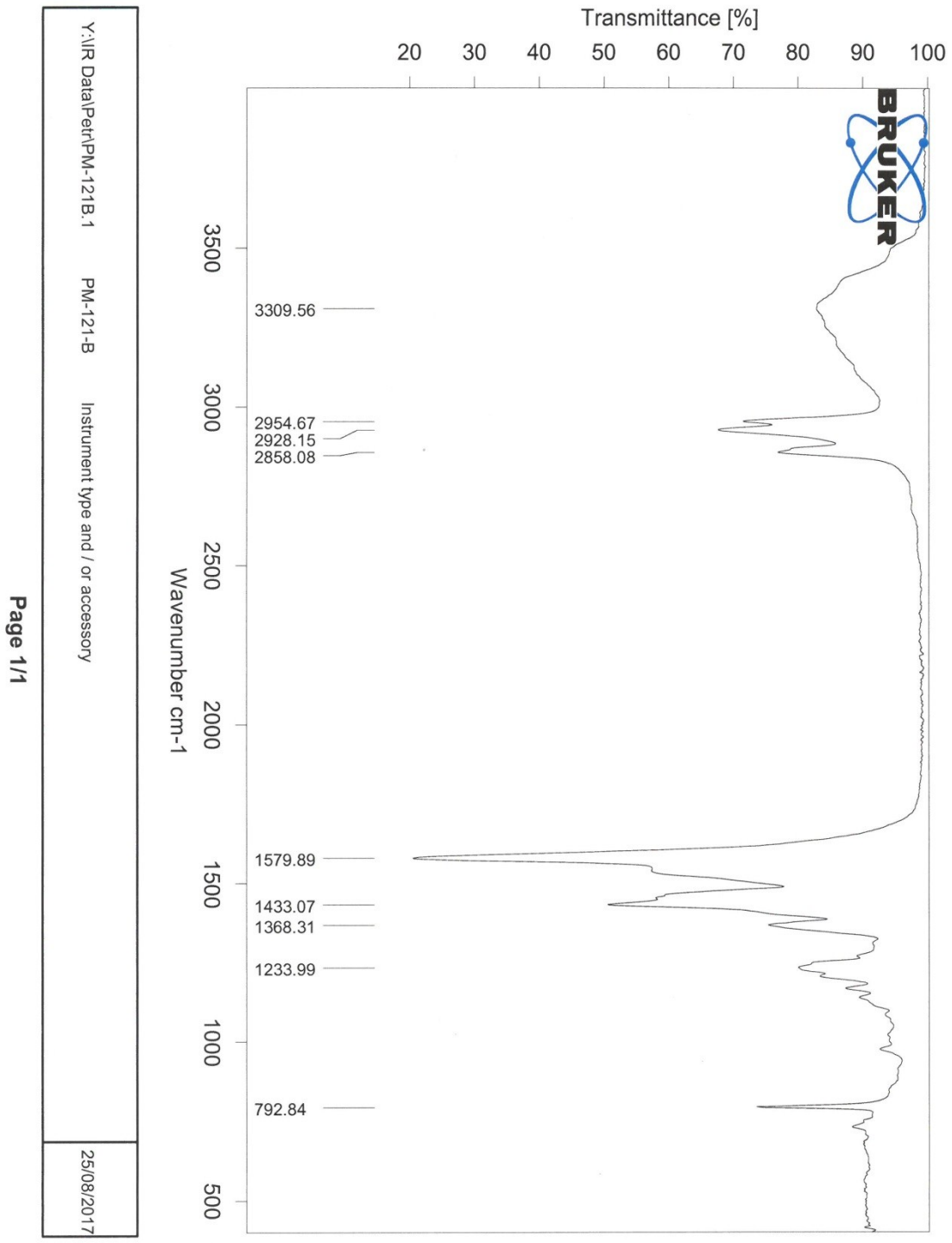
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2000
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1000
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Wavenumber cm-1
Y:\IR Data\Petr\PM-121B.1
PM-121-B
Instrument type and / or accessory
25/08/2017
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HRMS

Openlynx Report - Hunter_C
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File:Hunter_C246-2
Description:

Vial:1:6
Date:25-Aug-2017
Method:C:\MassLynx\Acc-mass.olp

Sample ID:PM-121
Time:16:34:15
Group:Hunter_C

Printed: Fri Aug 25 16:47:18 2017

Sample Report (continued):

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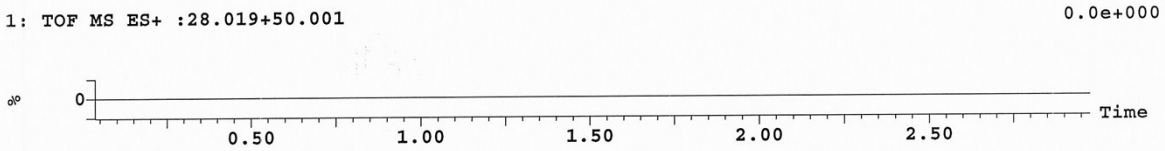

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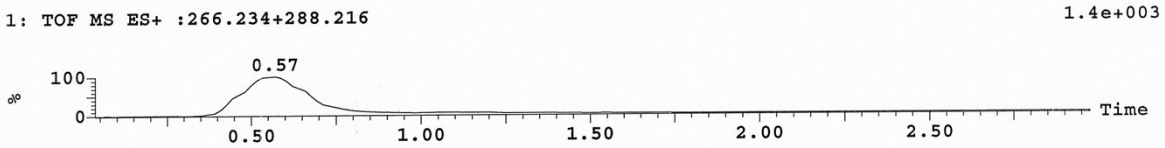

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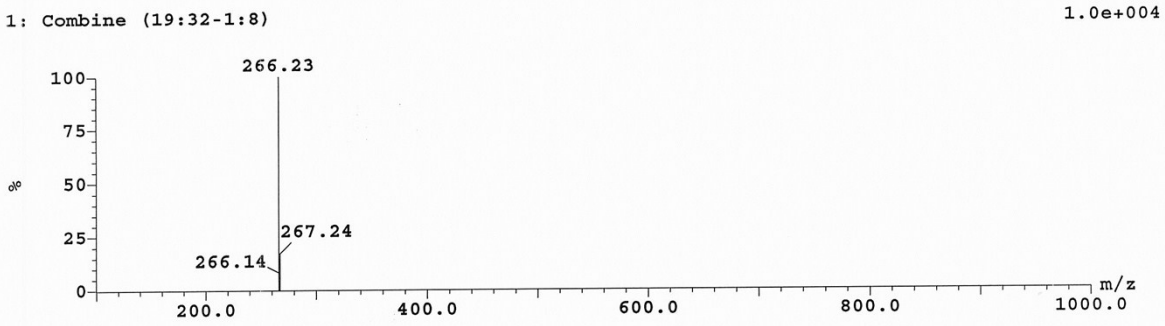
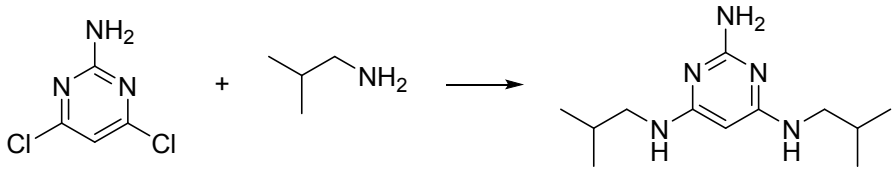


Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT
266.2348	266.2345	0.3	1.1	3.5	C14 H28 N5	2.6

N4,N6-diisobutylpyrimidine-2,4,6-triamine (2)



4,6-Dichloropyrimidin-2-amine (307 mg, 1.87 mmol) was flushed with nitrogen in MW vial. Isopropylamine (2 mL, 18.8 mmol) was added and the mixture was heated in microwave at 130 °C for 18 hours. The remaining amine was removed under reduced pressure and the residue was loaded on Celite. A combiflash of the residue on silica (EtOAc/MeOH: MeOH 0%→5%→10%) provided the title compound (392 mg, 88% yield) as a yellowish solid.

^{1}H NMR ($CDCl_3$, 400 MHz, 298 K): δ 4.79 (s, 1H), 4.57 (s, 2H), 4.46 (s, 2H), 3.00 – 2.93 (m, 4H), 1.90 – 1.78 (m, 2H), 0.95 (d, J = 6.5 Hz, 12H).
^{13}C NMR ($CDCl_3$, 101 MHz, 298 K): δ 164.4, 162.4, 71.9, 49.4, 28.3, 20.3.

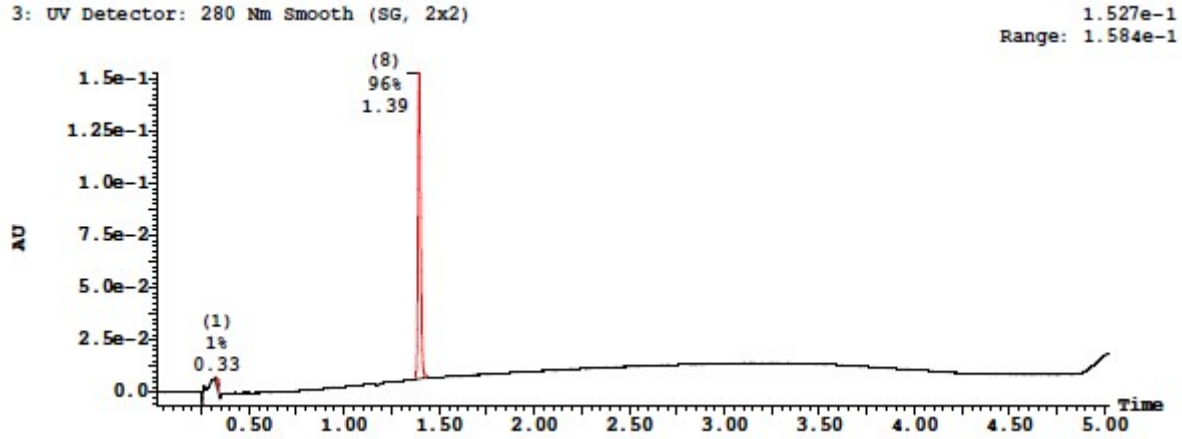
HR-MS (ESI): Calculated for $C_{12}H_{24}N_5$ $[M+H]^+$ 238.2032, found: 238.2035 (Δ = 1.3 ppm)

FT-IR (thin film): 3453, 3264, 3104, 2960, 2868, 2402, 1578, 1428 cm^{-1}.

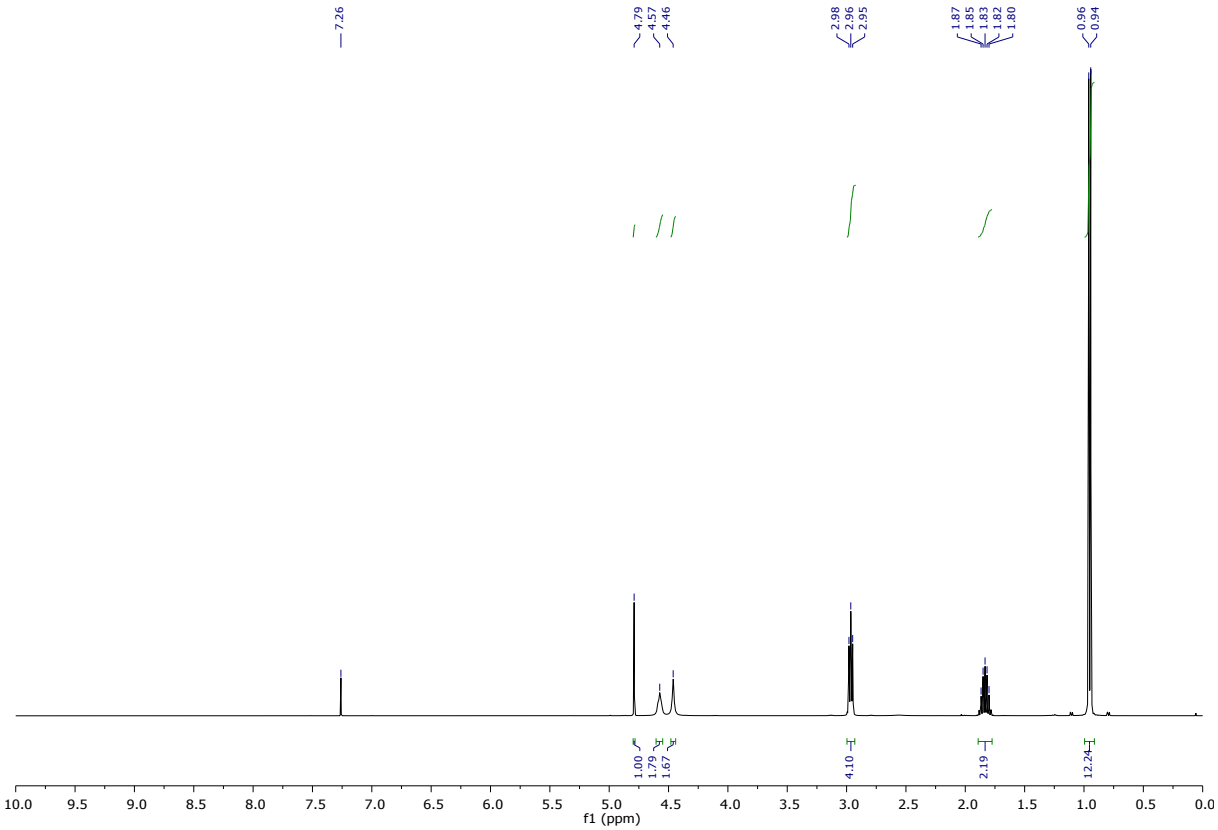
MP: 151 – 153 °C

LCMS Method: SLOW

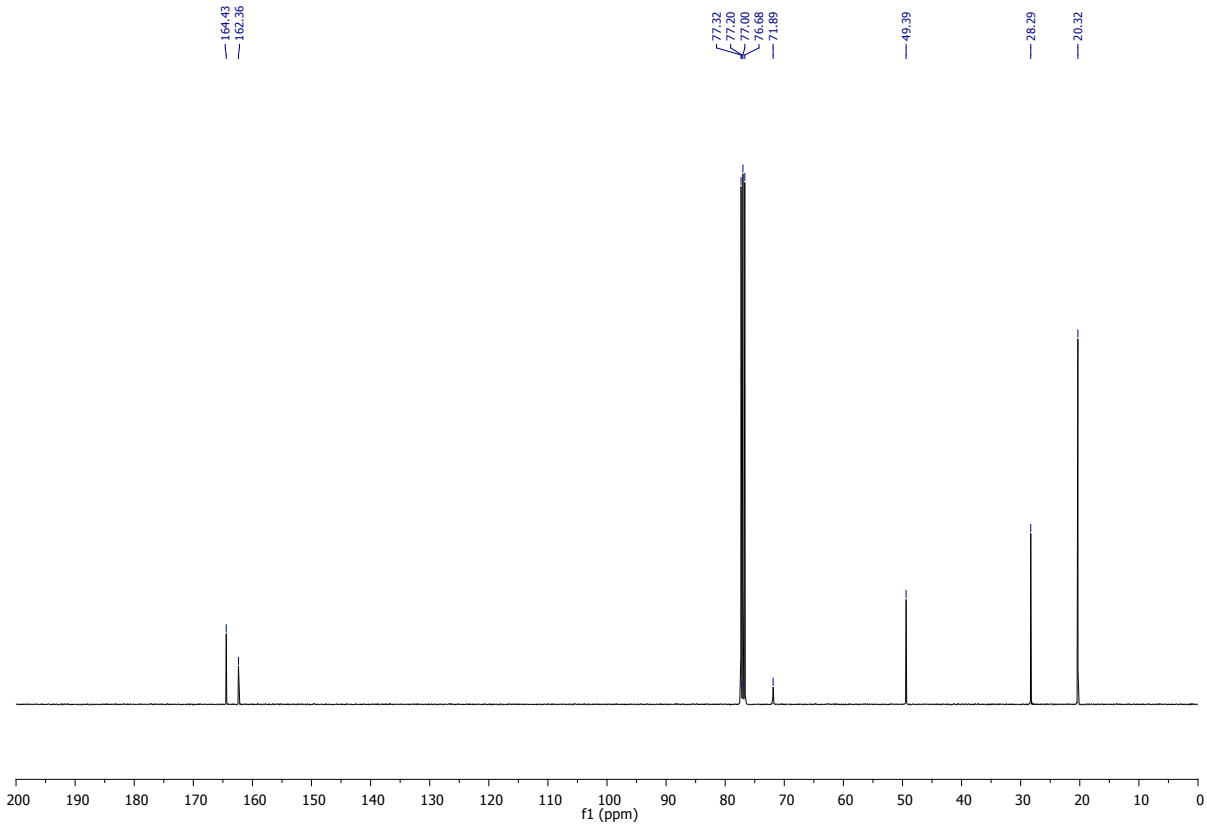
LCMS



^{1}H NMR



^{13}C NMR



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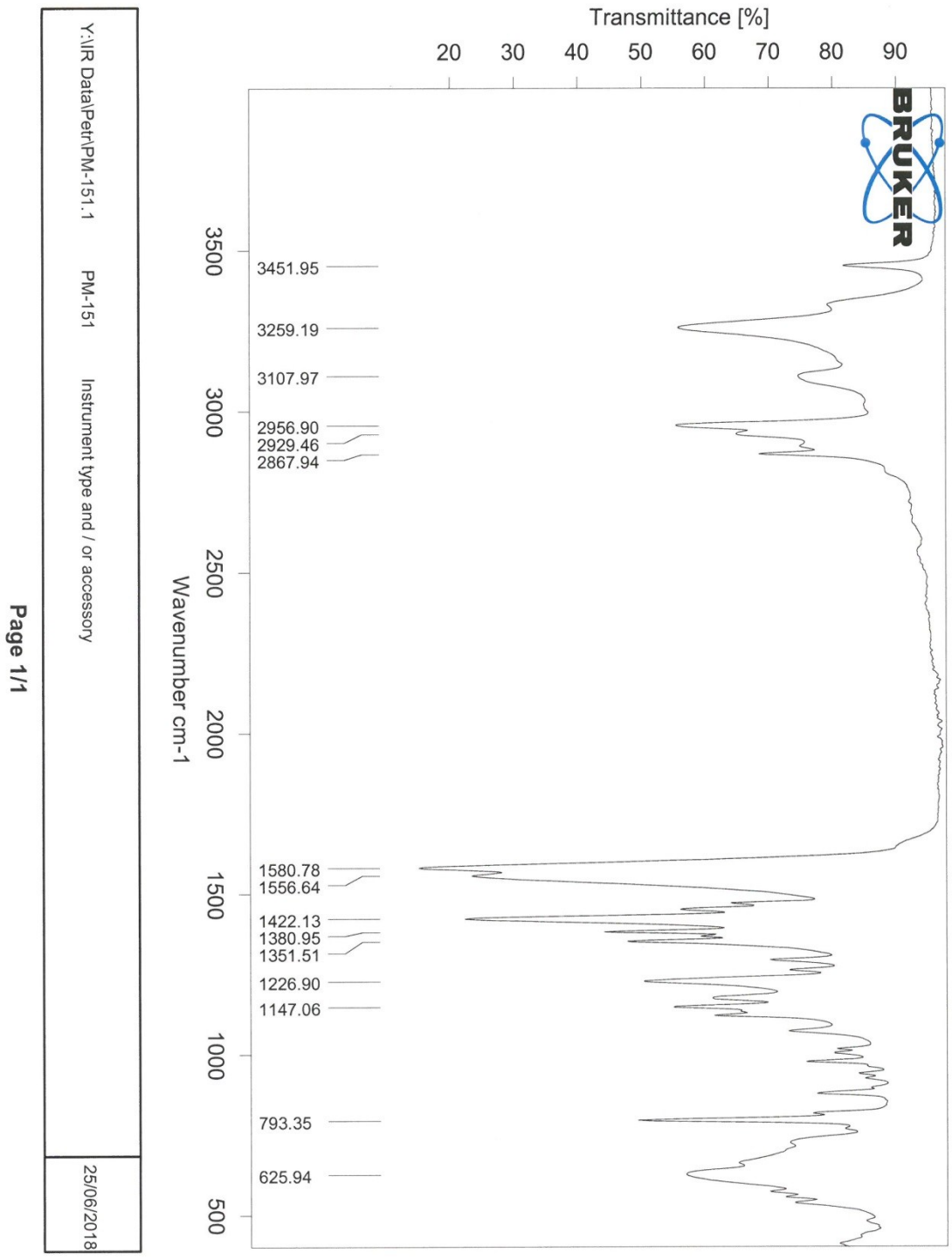
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Wavenumber cm-1
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PM-151
Instrument type and / or accessory
25/06/2018
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HR-MS

Openlynx Report - Hunter_C

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File:Hunter_C267-1 Date:12-Sep-2017 Time:17:26:20
Description: Method:C:\MassLynx\Acc-mass.olp Group:Hunter_C

Printed: Tue Sep 12 17:38:26 2017

Sample Report:

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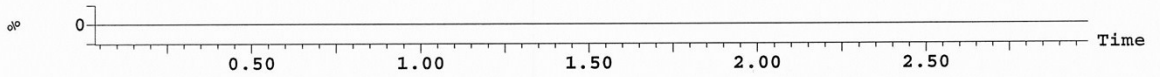
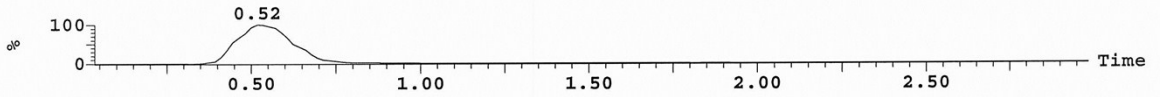


1: TOF MS ES+ :238.203+260.185 1.9e+003



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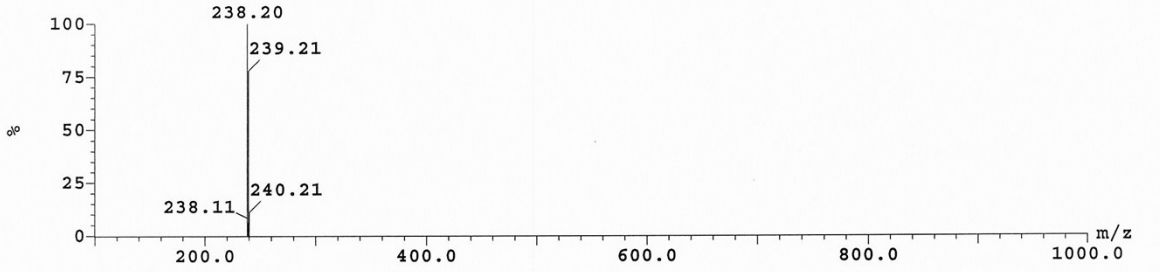
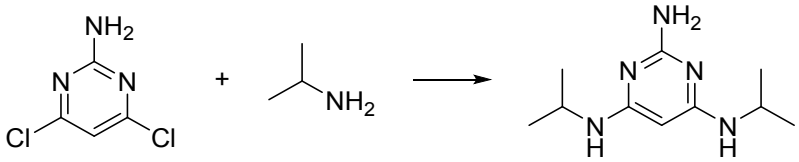


Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT
238.2035	238.2032	0.3	1.3	3.5	C12 H24 N5	3535.5

N4,N6-diisopropylpyrimidine-2,4,6-triamine (3)



4,6-Dichloropyrimidin-2-amine (317 mg, 1.89 mmol) was flushed with nitrogen in MW vial. Isopropylamine (2.3 mL, 1.6 g, 27 mmol) was added and the mixture was heated in microwave at 140 °C for 25 hours. A saturated solution of $NaHCO_3$ (20 mL) was added and the mixture was extracted with CH_2Cl_2/MeOH (10:1; 3 × 50 mL) and CH_2Cl_2 (50 mL). The organic phase was dried over $MgSO_4$, evaporated and loaded to Celite. A combiflash of the residue on silica (CH_2Cl_2/MeOH: MeOH 0%→10%) provided the title compound (334 mg, 84% yield) as a pale-orange solid.

^{1}H NMR (CD_3OD, 400 MHz, 298 K): δ 4.90 (s, 1H), 3.83 – 3.73 (m, 2H), 1.17 (d, J = 6.5 Hz, 12H).
^{13}C NMR ($CDCl_3$, 101 MHz, 298 K): δ 163.3, 162.5, 72.6, 42.7, 22.9.

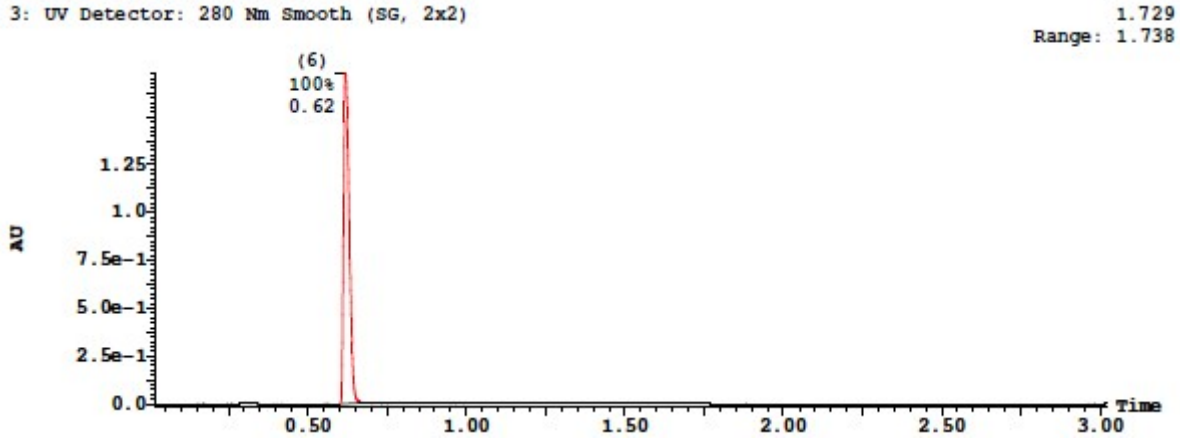
HR-MS (ESI): Required for $C_{10}H_{20}N_5$ $[M+H]^+$ 210.1719 , found: 210.1713 (Δ = 2.9 ppm).

FT-IR (thin film): 3314, 2968, 2930, 2872, 1572, 1521, 1494, 1365, 1337, 1192, 1125 cm^{-1}.

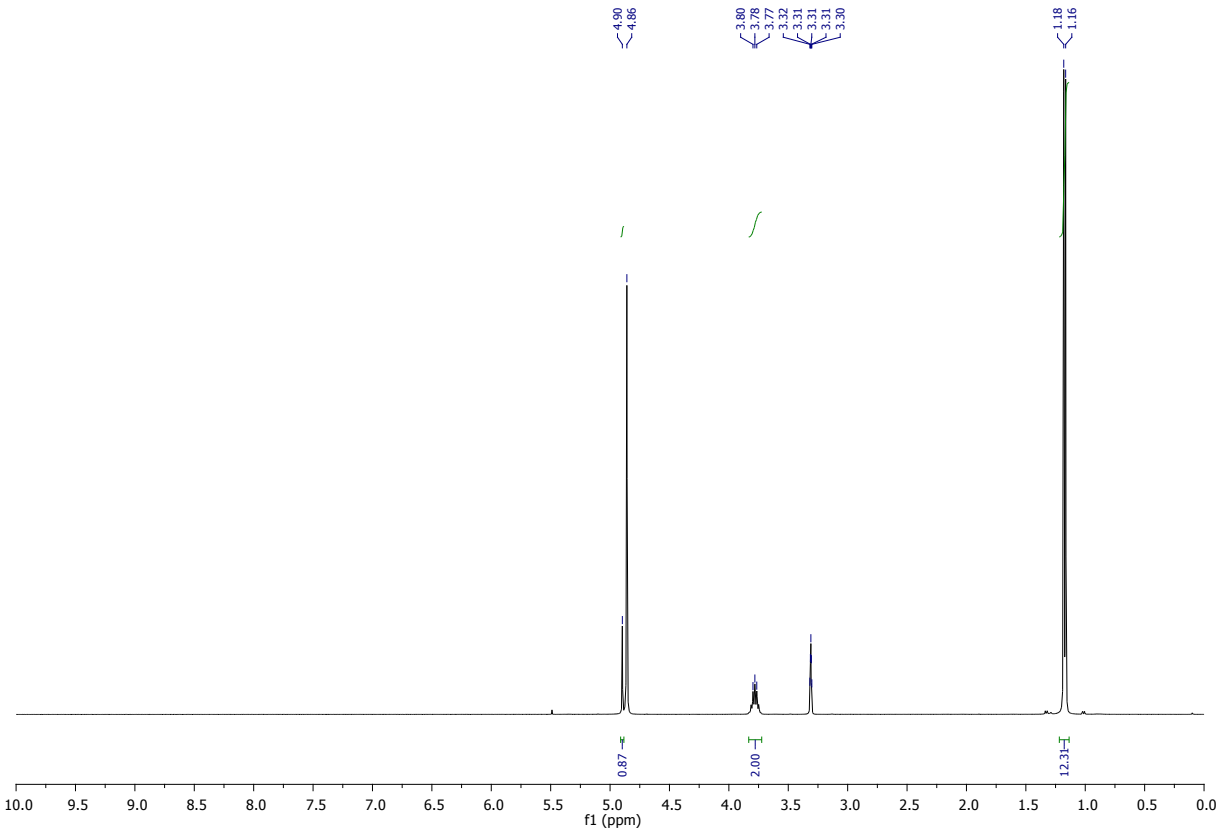
MP: 128 – 131 °C

LCMS Method: FAST

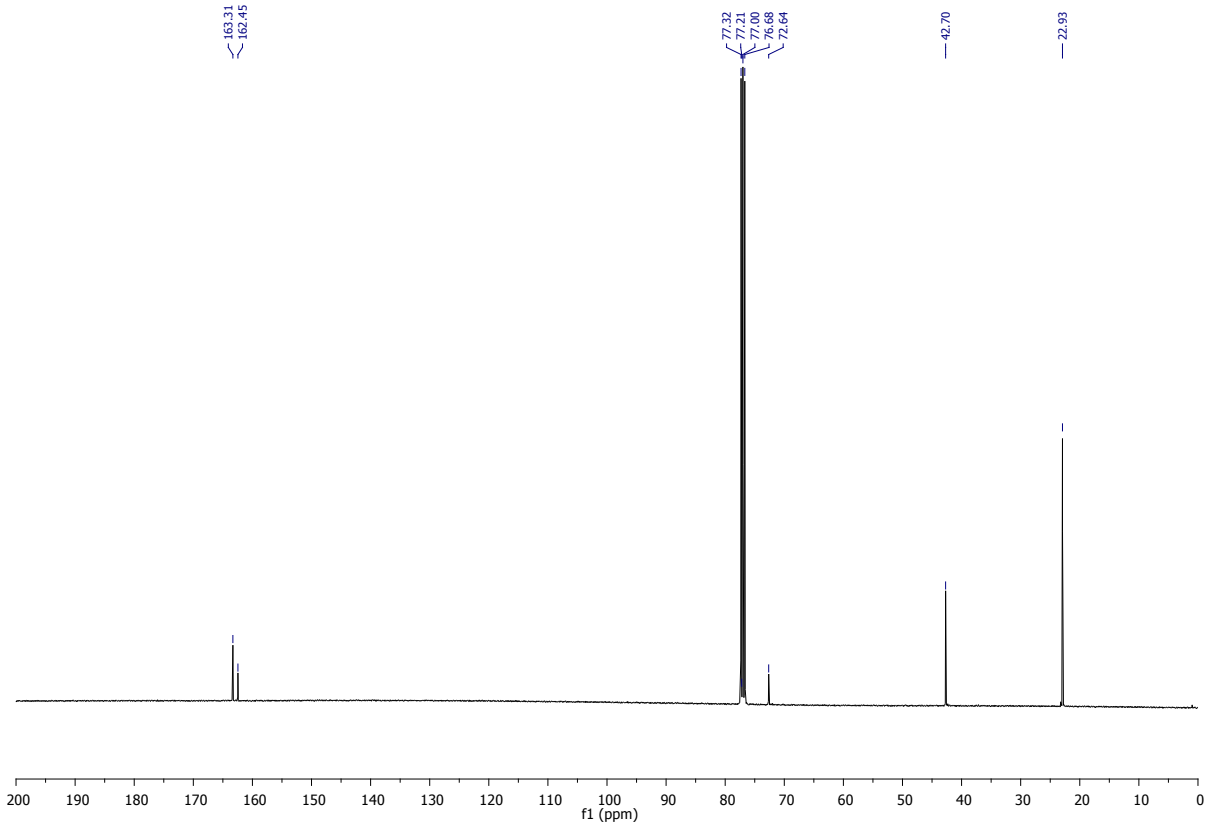
LCMS



^{1}H NMR



^{13}C NMR



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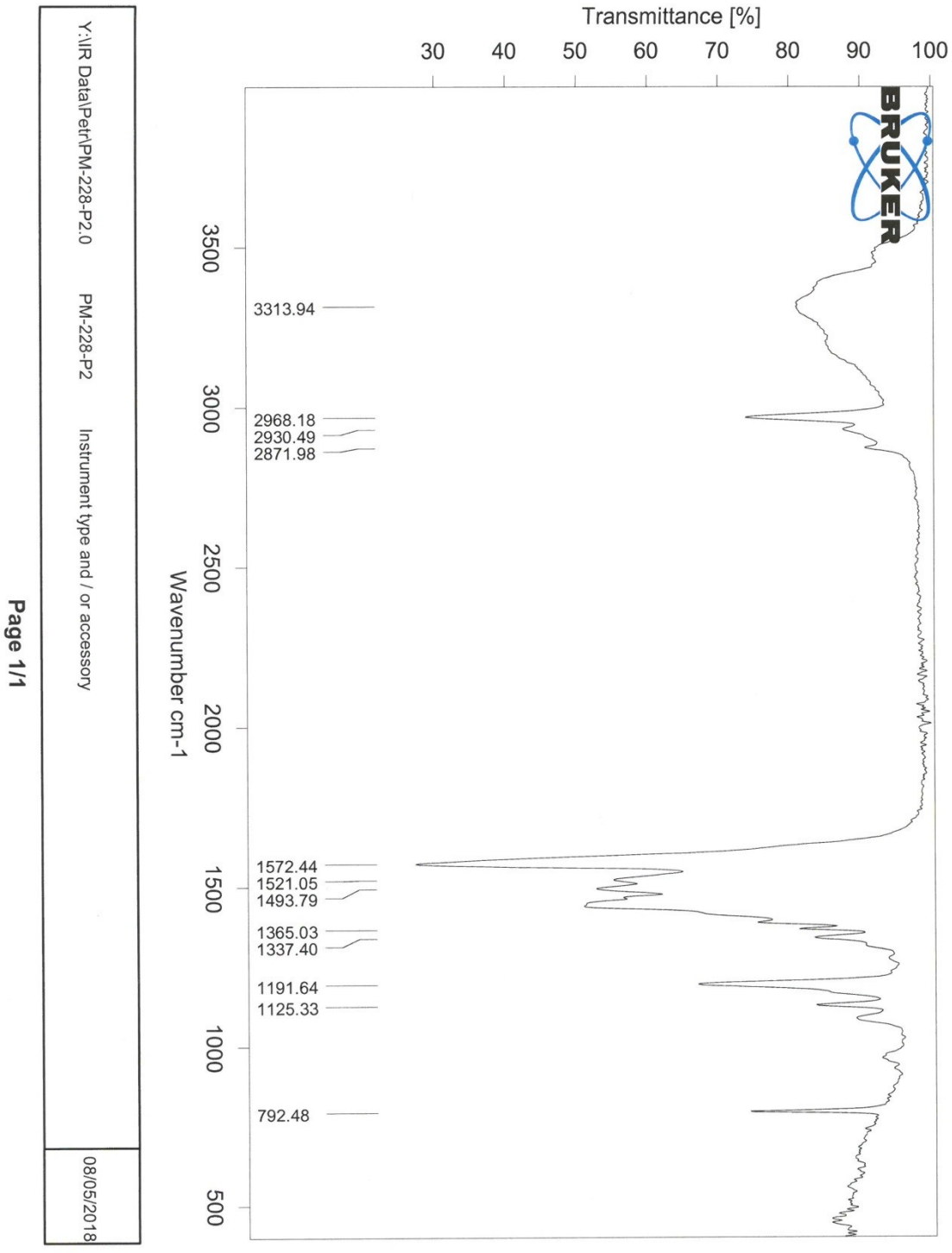
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1337.40
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1000
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Wavenumber cm-1
Y:\IR Data\Petr\PM-228-P2.0
PM-228-P2
Instrument type and / or accessory
08/05/2018
Page 1/1

HR-MS

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File:MassSpecService3446-1 Date:12-Jun-2018 Time:10:41:06
Description: Method:C:\MassLynx\Acc-mass.olp Group:MassSpecService

Printed: Tue Jun 12 10:53:43 2018

Sample Report:

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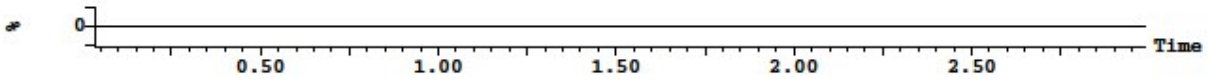

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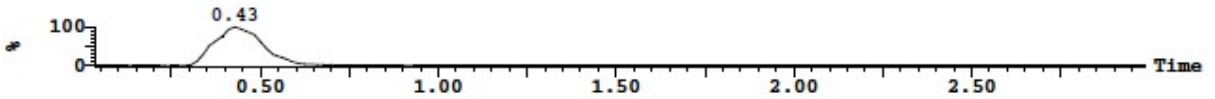

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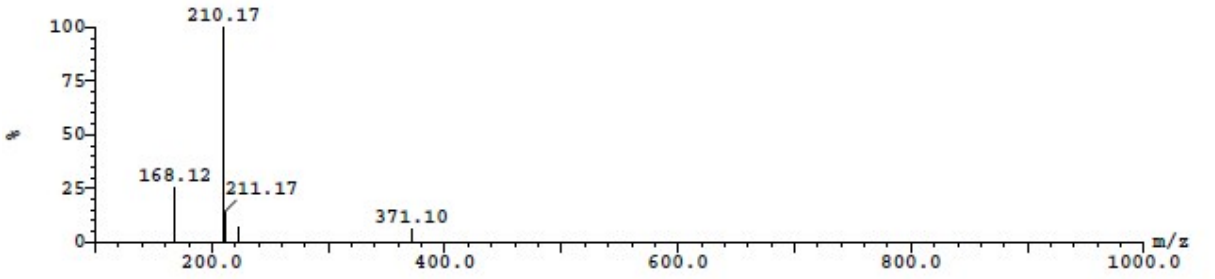
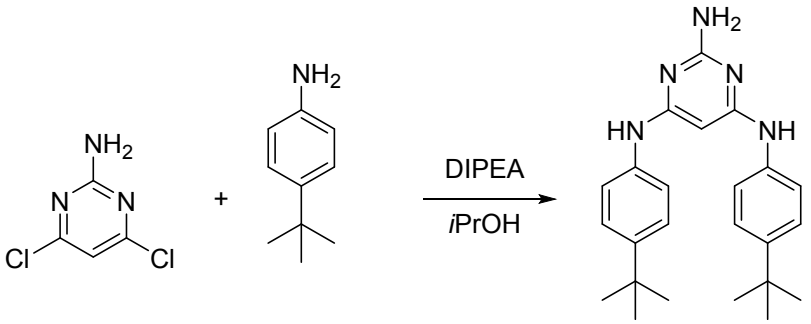


Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT
210.1713	210.1719	-0.6	-2.9	3.5	C10 H20 N5	4.2

N4,N6-bis(4-(tert-butyl)phenyl)pyrimidine-2,4,6-triamine (4)



4,6-Dichloropyrimidin-2-amine (178 mg, 1.06 mmol) was flushed with nitrogen in MW vial. N,N-diisopropylethylamine (0.5 mL, 2.48 mmol), isopropyl alcohol (1.0 mL) and 4-tertbutylaniline (0.6 mL, 562 mg, 3.77 mmol) were added and the mixture was heated in microwave at 150 °C for 1 hours. Then, additional 4-tertbutylaniline (0.5 µL, 467 mg, 3.14 mmol) was added under nitrogen atmosphere and the mixture was heated in microwave at 170 °C for 10 hours. A saturated solution of $NaHCO_3$ (20 mL) and water (20 mL) were added and the mixture was extracted with CH_2Cl_2/MeOH (10:1; 2 × 50 mL) and CH_2Cl_2 (2 × 50 mL). The organic phase was dried over $MgSO_4$, evaporated and loaded to Celite. A combiflash of the residue on silica (CH_2Cl_2/MeOH: MeOH 0%→10%) provided the title compound (294 mg, 71% yield) as a pale-pink solid.

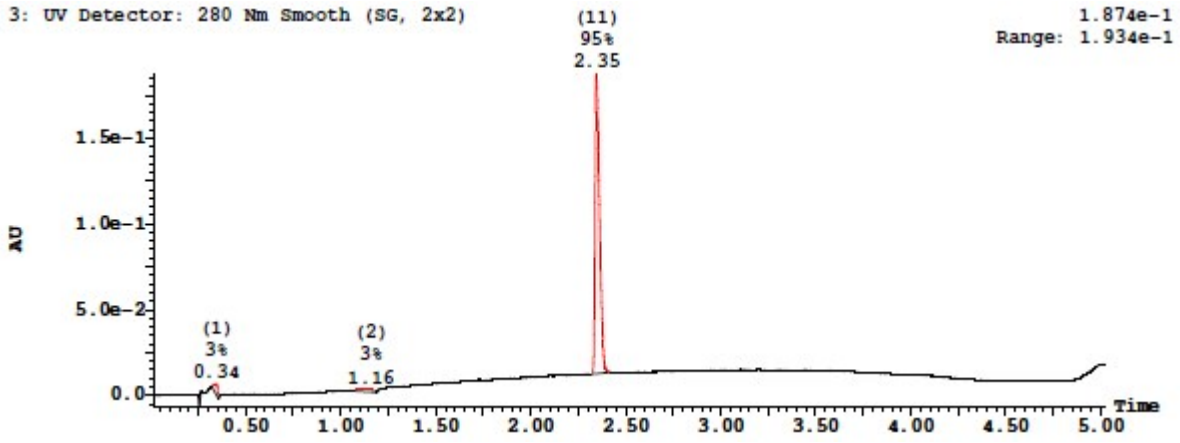
^{1}H NMR (d^6-DMSO, 400 MHz, 298 K): δ 8.52 (s, 2H), 7.44 (d, J = 8.5 Hz, 4H), 7.25 (d, J = 8.5 Hz, 4H), 5.80 (s, 2H), 5.50 (s, 1H), 1.26 (s, 18H).

MP: 281 – 284 °C

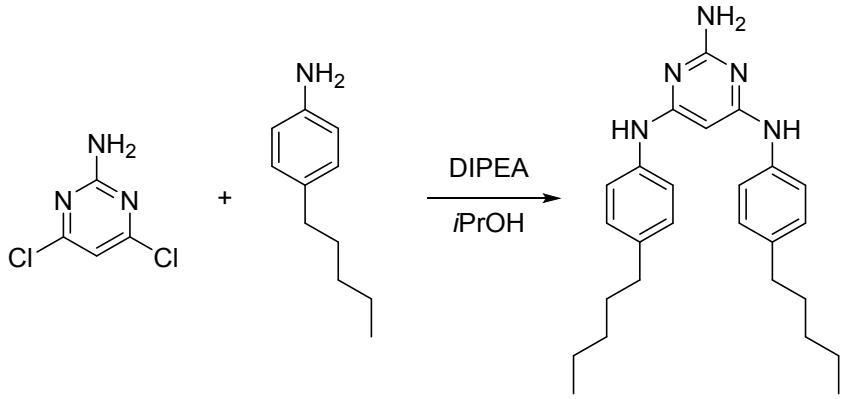
LCMS Method: SLOW

The observed data were in agreement with the reported values.[1]

LCMS



N4,N6-bis(4-pentylphenyl)pyrimidine-2,4,6-triamine (5)



4,6-Dichloropyrimidin-2-amine (158 mg, 0.944 mmol) was flushed with nitrogen in MW vial. N,N-diisopropylethylamine (400 µL, 2.30 mmol), isopropyl alcohol (1.1 mL) and 4-pentylaniline (500 µL, 460 mg, 2.81 mmol) were added, and the mixture was heated in microwave at 150 °C for 2 hours. Then, additional 4-pentylaniline (500 µL, 460 mg, 2.81 mmol) was added under nitrogen atmosphere and the mixture was heated in microwave at 160 °C for 16 hours. A saturated solution of $NaHCO_3$ (20 mL) and water (20 mL) were added and the mixture was extracted with CH_2Cl_2/MeOH (10:1; 2 × 50 mL) and CH_2Cl_2 (2 × 50 mL). The organic phase was dried over $MgSO_4$, evaporated and loaded to Celite. A combiflash of the residue on silica (CH_2Cl_2/MeOH: MeOH 0%→10%) provided the title compound (300 mg, 76% yield) as a white solid.

^{1}H NMR ($CDCl_3$, 400 MHz, 298 K): δ 7.15 – 7.09 (m, 8H), 6.46 (s, 2H), 5.61 (s, 1H), 4.64 (s, 2H), 2.64 – 2.47 (t, J = 7.5 Hz, 4H), 1.62 – 1.55 (m, 4H), 1.38 – 1.26 (m, 8H), 0.89 (t, J = 7.0 Hz, 6H).
^{13}C NMR ($CDCl_3$, 101 MHz, 298 K): δ 162.8, 162.6, 138.7, 136.6, 129.0, 122.2, 75.9, 35.3, 31.4, 31.2, 22.5, 14.0.

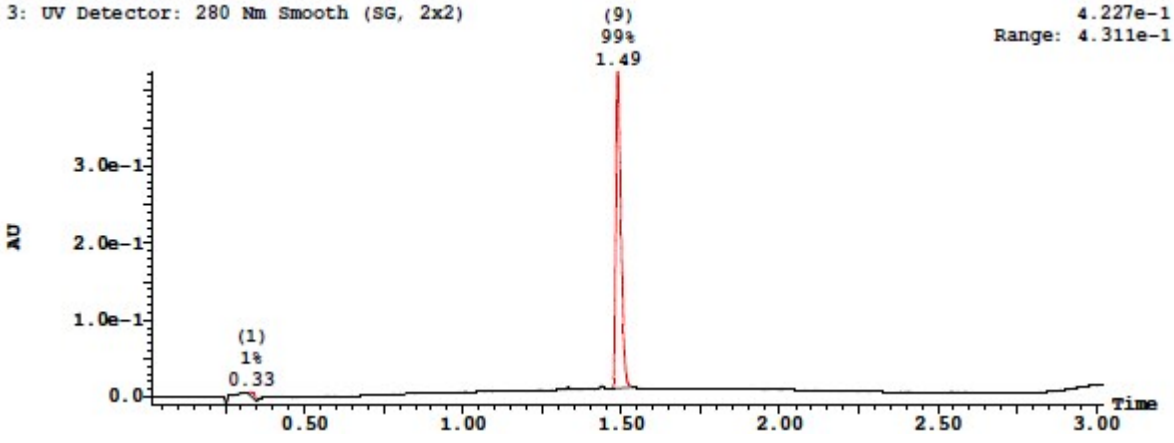
HR-MS (ESI): Required for $C_{26}H_{36}N_5$ $[M+H]^+$ 418.2971, found: 418.2959 (Δ = 2.9 ppm).

FT-IR (thin film): 3391, 3319, 3181, 2955, 2924, 2854, 1574, 1507, 1412, 1278, 1240 cm^{-1}.

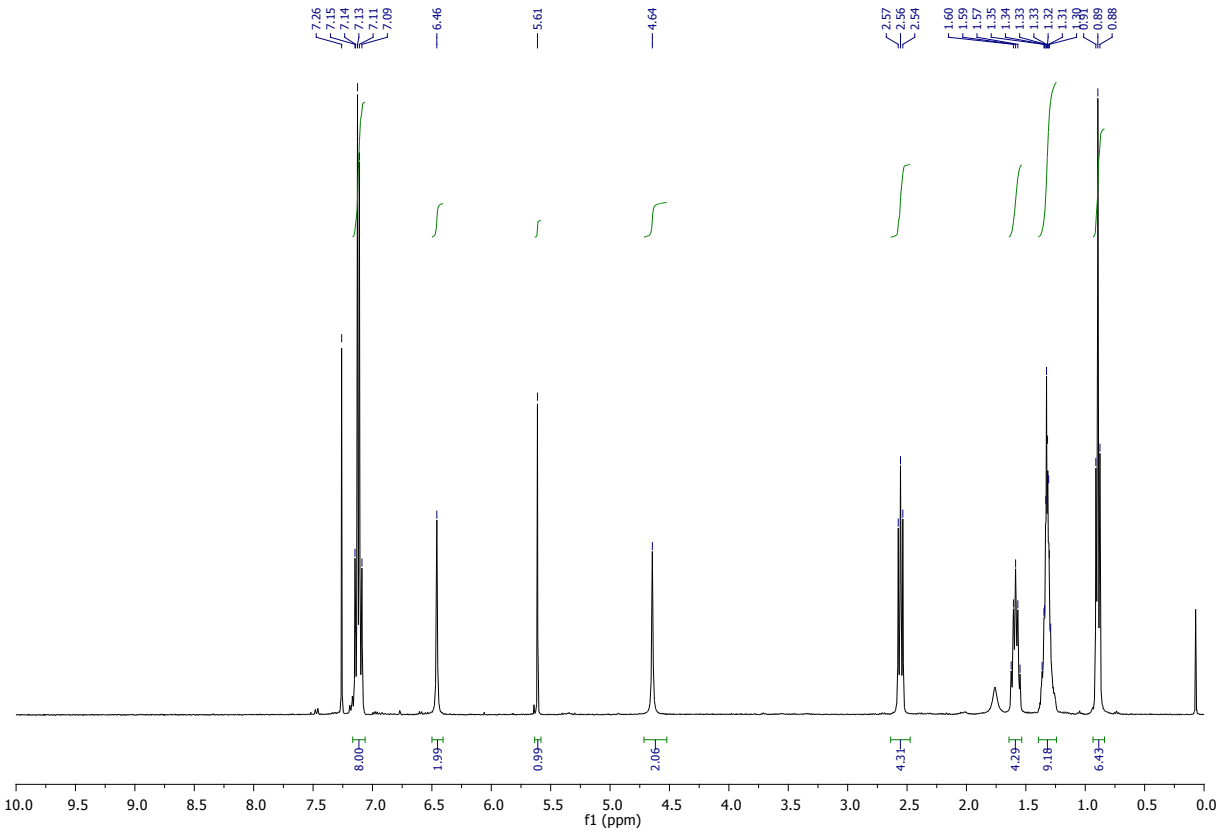
MP: 174 – 176 °C

LCMS Method: FAST

LCMS



^{1}H NMR



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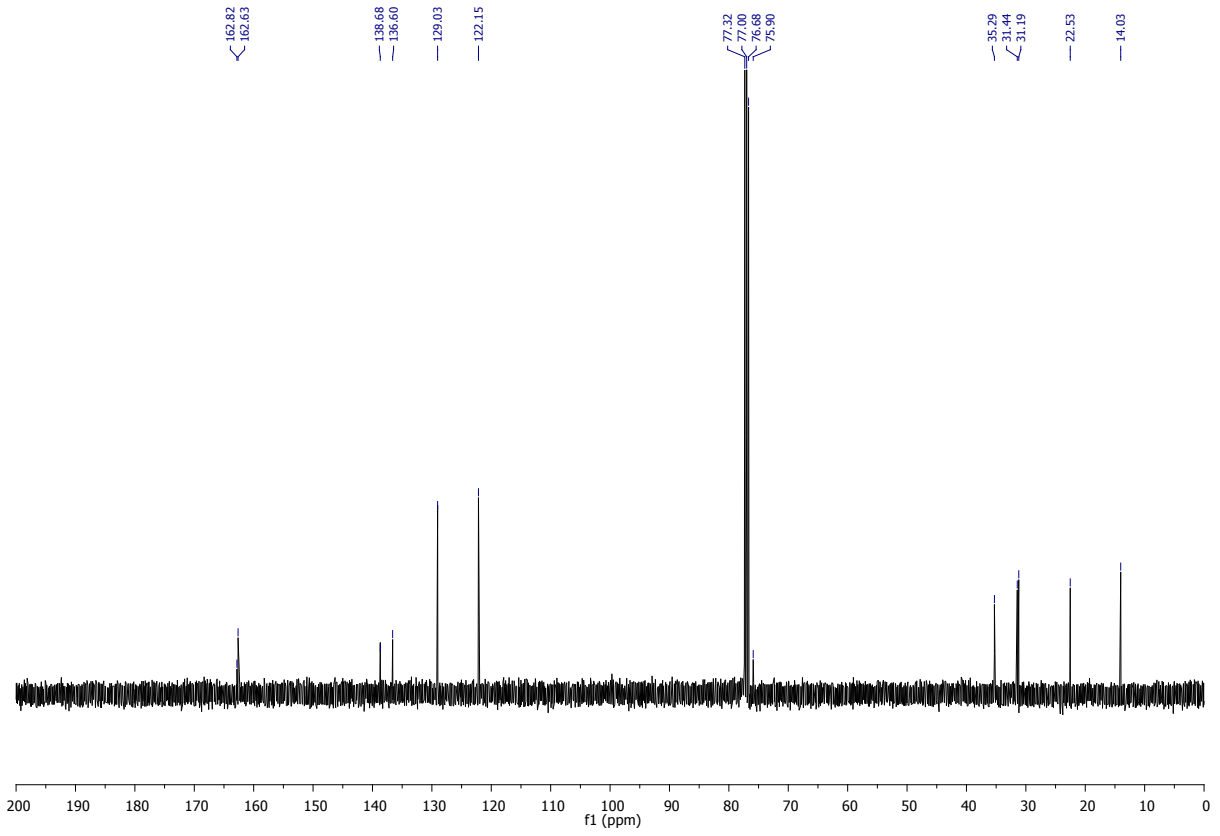
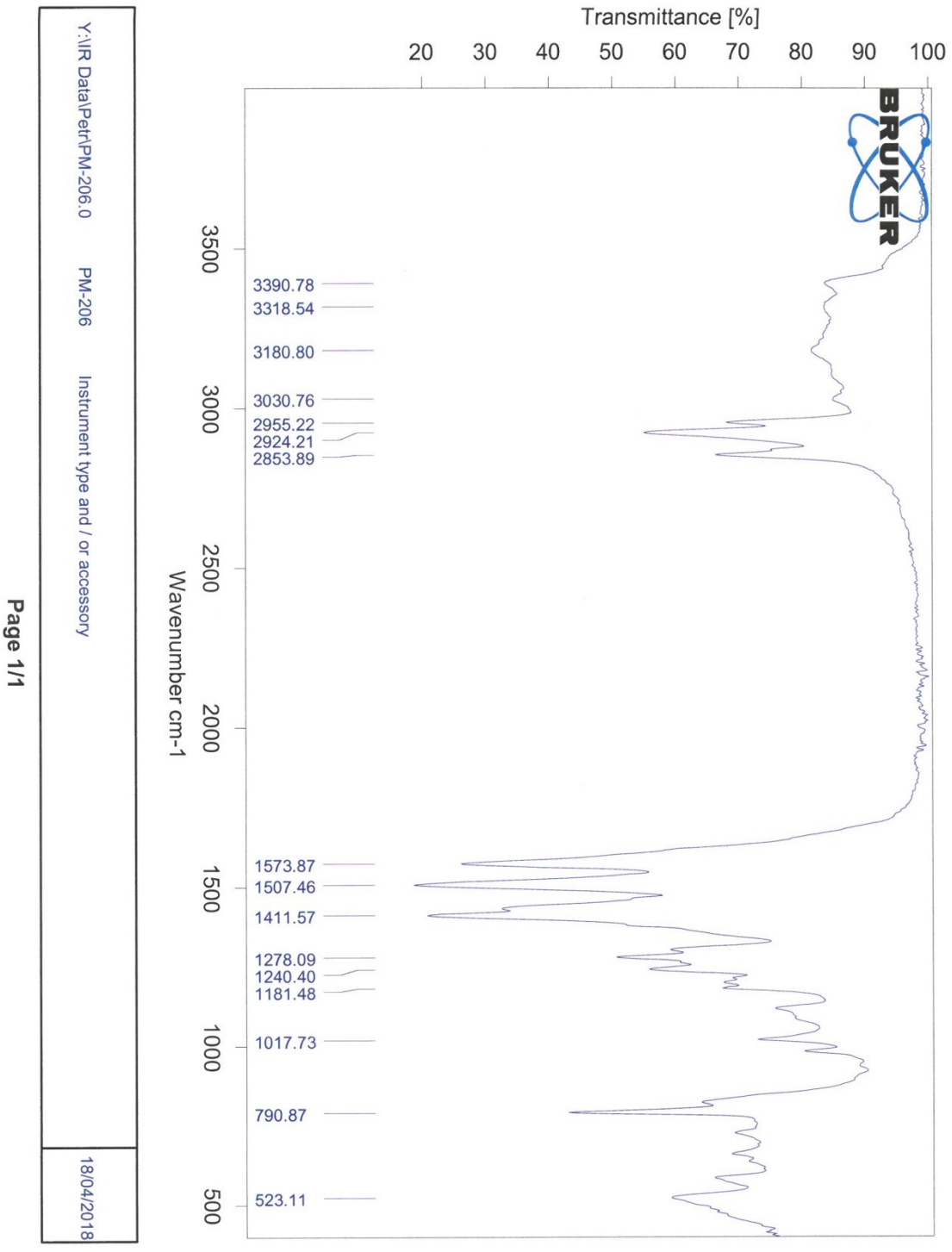


IR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50
60
70
80
90
100
BRUKER
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1500
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Wavenumber cm-1
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3318.54
3180.80
3030.76
2955.22
2924.21
2853.89
1573.87
1507.46
1411.57
1278.09
1240.40
1181.48
1017.73
790.87
523.11
Y:\IR Data\Petr\PM-206.0
PM-206
Instrument type and / or accessory
18/04/2018
Page 1/1

HR-MS

Openlynx Report - Hunter_C

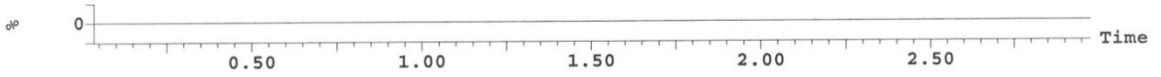
Sample: 1 Vial:1:15 Sample ID:PM-206
File:Hunter_C310-1 Date:18-Apr-2018 Time:11:42:09
Description: Method:C:\MassLynx\Acc-mass-5ul.olp Group:Hunter_C

Printed: Wed Apr 18 11:58:20 2018

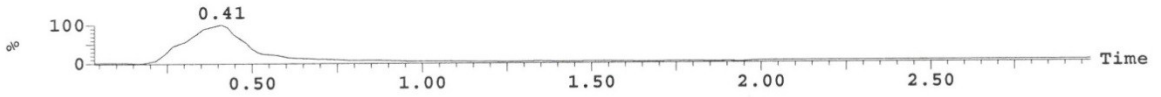
Sample Report:

Sample 1 Vial 1:15 ID PM-206 File Hunter_C310-1 Date 18-Apr-2018 Time 11:42:09

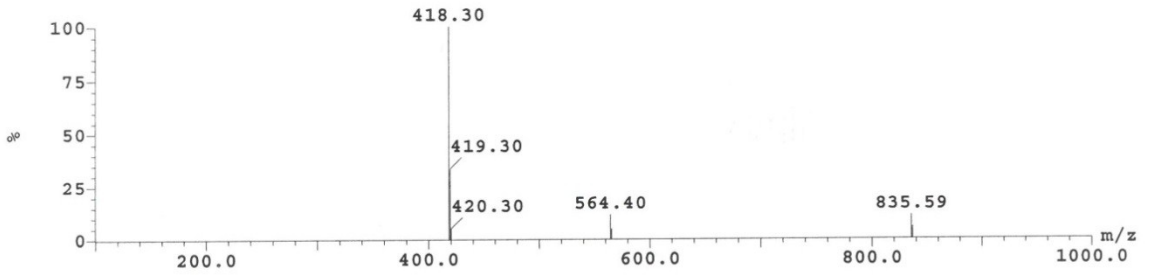
1: TOF MS ES+ :28.019+50.001 0.0e+000



1: TOF MS ES+ :418.297+440.279 1.9e+002



1: Combine (16:32-1:10) 1.2e+003



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT
418.2959	418.2971	-1.2	-2.9	11.5	C26 H36 N5	0.7
418.2959	418.2962	-0.3	-0.7	5.0	C9 H30 N20	52.4

4. X-ray crystallography

The single crystals **2**·HCl and **2**·HI were obtained by slow vapour diffusion of the HCl or HI, respectively, from their concentrated aqueous solutions into dichloromethane solutions of **2** at 5^0C. Single-crystal X-ray data were collected on a Bruker D8-QUEST PHOTON-100 instrument equipped with an Incoatec Cu microsource (lambda = 1.54 Å). Data collection and processing was carried out using the APEX3 software suite (Bruker, 2016). Structures were solved using SHELXT[2] and refined using SHELXL.[3]

2·HCl

These crystals were difficult to handle. They appeared to be damaged at the lab's usual analysis temperature of 180 K. So they were examined at 220 K, where the diffraction pattern looked better. The data were still weak and have been truncated at 0.95 Å resolution. The structure contains large voids, but there is no significant electron density to indicate solvent molecules. Applying SQUEEZE has little effect. Probably there is solvent loss, consistent with the difficult sample handling.

Formula	$C_{12}H_{25}N_5Cl_2$		
Temperature / K	220		
Space Group	I 4_1a/		
Cell Lengths / Å	**a** 17.7220(6)	**b** 17.7220(6)	**c** 28.3433(14)
Cell Angles / °	**α** 90	**β** 90	**γ** 90
Cell Volume / Å³	8901.76		
Z	16		
R factor / %	7.4		

2·HI

Correlated disorder of iPr groups over two orientations

Formula	$C_{12}H_{24}N_5I$		
Temperature / K	180		
Space Group	$P\bar{1}$		
Cell Lengths / Å	**a** 8.3581(5)	**b** 7357(5)	**c** 12.2791(8)
Cell Angles / °	**α** 104.472	**β** 102.631(5)	**γ** 104.192(4)
Cell Volume / Å³	803.308		
Z	2		
R factor / %	6.55		

5. Transmembrane transport experiments

HPTS experiment

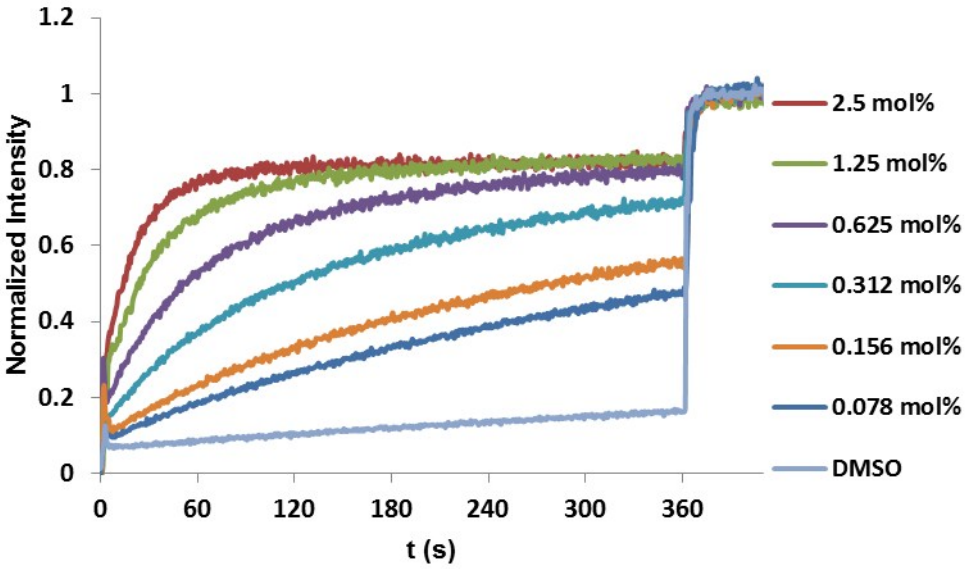
The LUVs were prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC chloroform solution). The solvent was evaporated without heating and the residue dried for 2h under high vacuum. The resulting thin film was hydrated in 400 mL of buffer (10 mM phosphate buffer, pH 6.4, 200 mM KCl or 133 mM K_2SO_4) containing 1 mM 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS). During hydration, the suspension was subjected to five freeze-thaw cycles (liquid nitrogen, water at room temperature). The obtained suspension was extruded 21 times through a 200 nm polycarbonate membrane. The LUV suspension was separated from extravesicular HPTS dye by size exclusion chromatography using prepacked Sephadex G-25M columns (prequilibrated with 200 mM KCl or 133 mM K_2SO_4 in 10 mM phosphate buffer, pH 6.4, as mobile phase). The obtained LUV solution was diluted to the final concentration of 1 mM and used as stock solution within the day.

Fluorescence spectroscopic data were recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent). We used a ratio data collection method.[4] 500 μL of stock vesicle solution was placed into a quartz fluorimetric cell. The emission of HPTS at 510 nm was monitored at two excitation wavelengths (405 and 460 nm) simultaneously. Prior to the experiment 2.5 μL of compound was inject from a stock solution in DMSO at the corresponding concentration. At t = 0s a corresponding volume of aqueous NaOH (0.1 M) was injected in order to increase the pH of the bulk solution to 7.4. (volume of needed NaOH solution was predetermined for each batch of vesicles using a SevenCompact pH/Ion S220 pH meter) Change in fluorescence was registered for 360s after which the vesicles are lysed with 10 μL 5% aqueous Triton X-100 solution to eqauilibrate the intra and extravesicular solution. Each compound was measured 3 times.

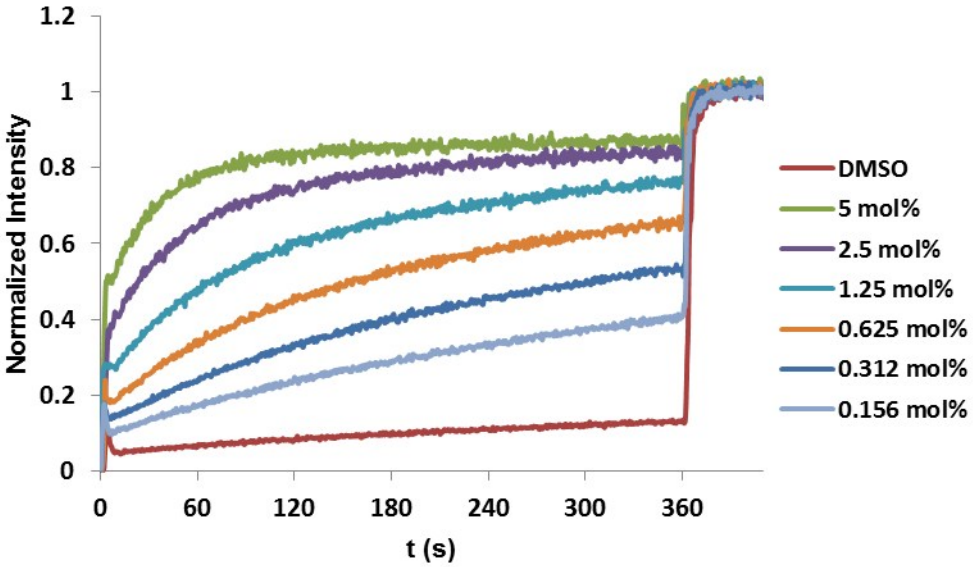
Lucigenin experiment

The LUVs were prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC chloroform solution). The solvent was evaporated without heating and the residue dried for 2h under high vacuum. The resulting thin film was hydrated in 400 mL of buffer (10 mM phosphate buffer, pH 7.2, 100 mM $NaNO_3$) containing 2 mM lucigenin. The suspension was briefly sonicated and subjected to five freeze-thaw cycles (liquid nitrogen, water at room temperature). The obtained suspension was extruded 21 times through a 200 nm polycarbonate membrane. The LUV suspension was separated from extravesicular lucigenin dye by size exclusion chromatography using prepacked Sephadex G-25M columns (prequilibrated with 100 mM $NaNO_3$). The obtained LUV solution was diluted to the final concentration of 1 mM and used as stock solution within the day.

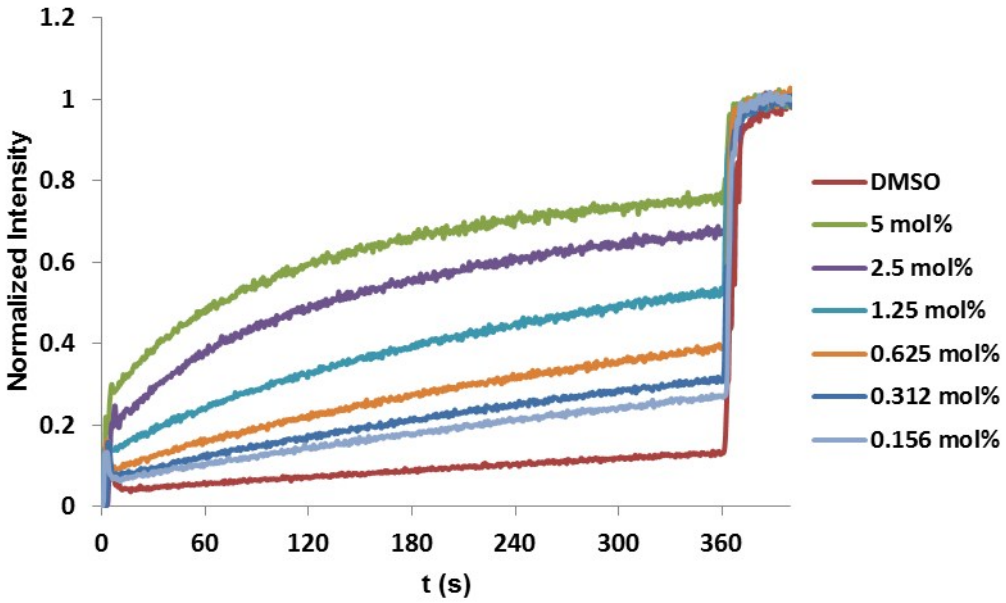
Fluorescence spectroscopic data were recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent). 500 μL of stock vesicle solution was placed into a quartz fluorimetric cell. The emission of lucigenin was monitored at 506 nm (λ_{ex}= 455 nm) Prior to the experiment 2.5 μL of compound was inject from a stock solution in DMSO and left to equilibrate for 3 min. At t = 0s a 5 uL of NaCl (2 M) solution was injected in order to initiate Cl^- transport. Change in fluorescence was registered for 330s after which the vesicles are lysed with 5 μL 5% aqueous Triton X-100 solution to equilibrate the intra and extravesicular solution.



ESI-Figure 2. Concentration dependent fluorescence assay of compound **1**



ESI-Figure 3. Concentration dependent fluorescence assay of compound **2**



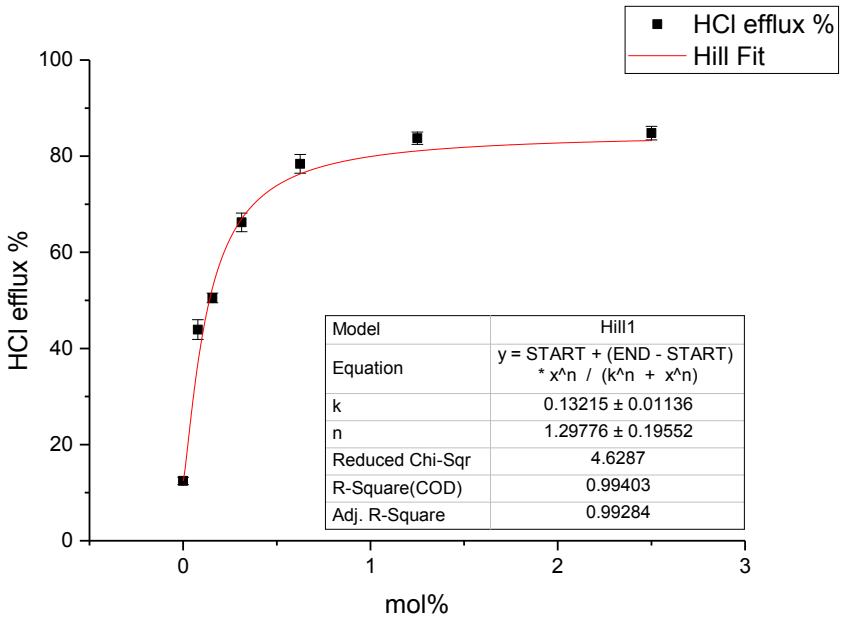
ESI-Figure 4. Concentration dependent fluorescence assay of compound **3**

Hill analysis

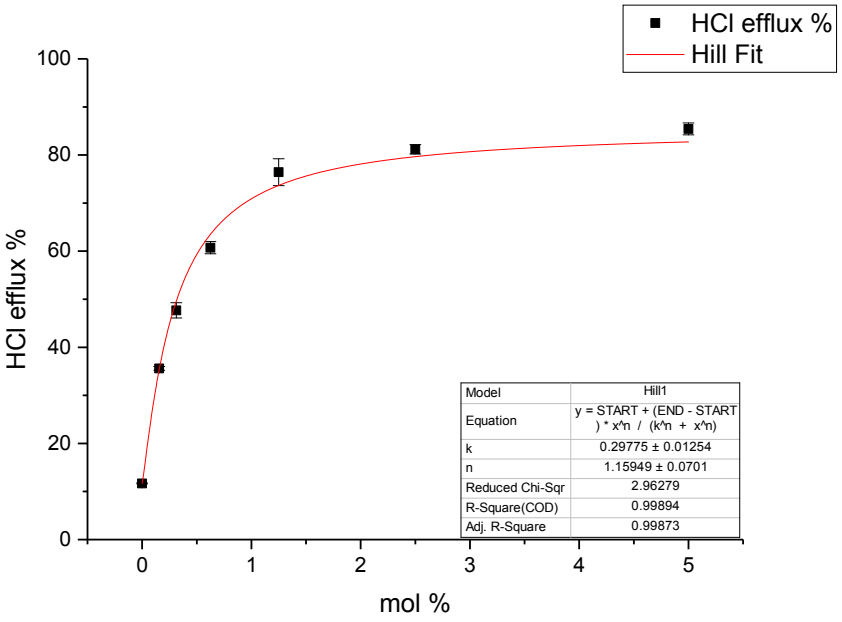
The anion flux (%) at 270s after the addition of the compound is plotted as a function of the compounds concentration. Using OriginPro 2017 the data points are then fitted to the Hill equation (1):

$$y = V_{max}\frac{x^n}{EC_{50} + x^n} \quad (1)$$

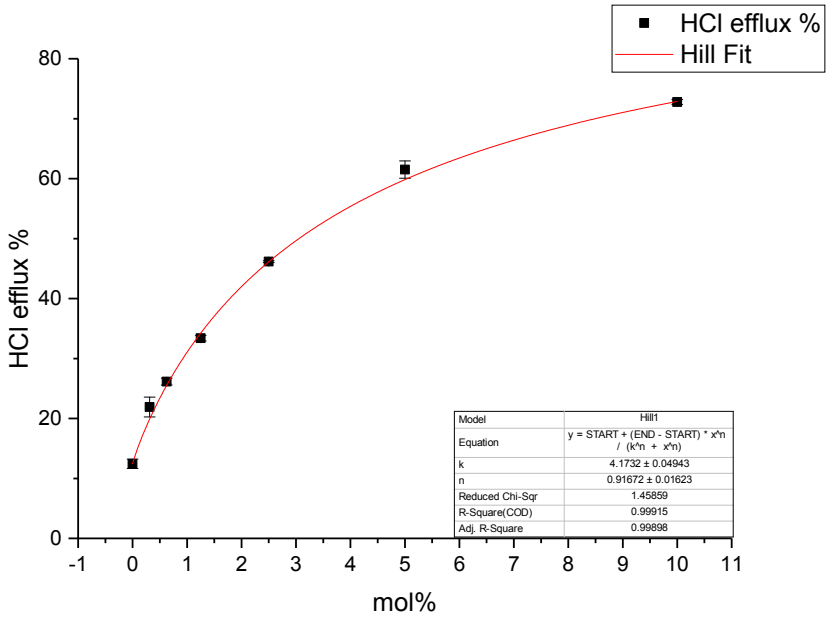
where y is the chloride influx at 270 s (%) and is x the carrier concentration (mol% carrier to lipid), V_{max} is the maximum efflux possible, n is the Hill coefficient and EC_{50} is the carrier concentration needed to reach $V_{max}/2$.



ESI-Figure 5. Hill plot analysis of compound **1**



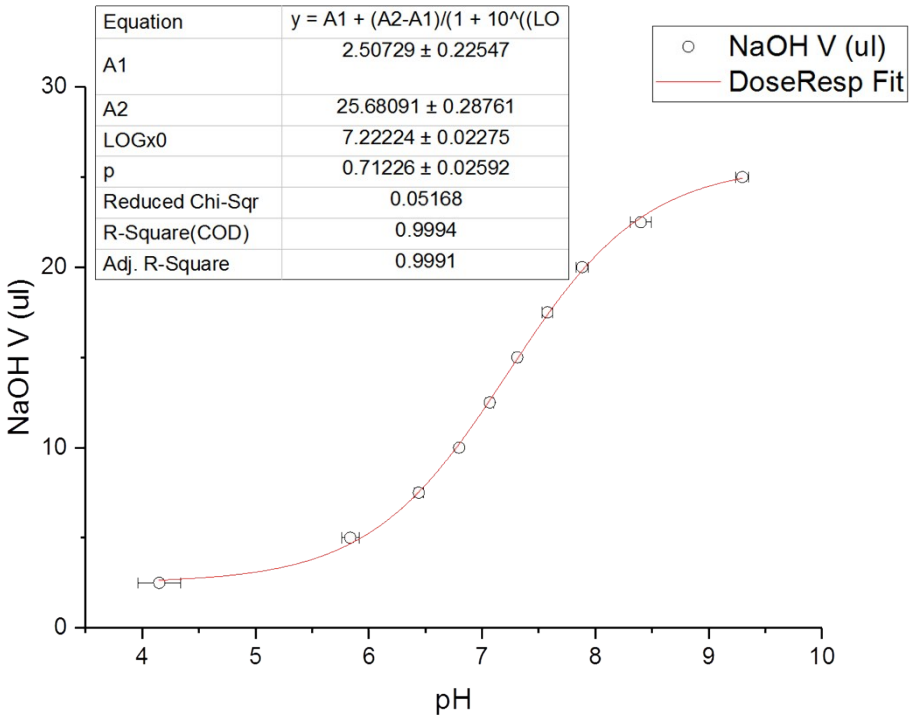
ESI-Figure 6. Hill plot analysis of compound **2**



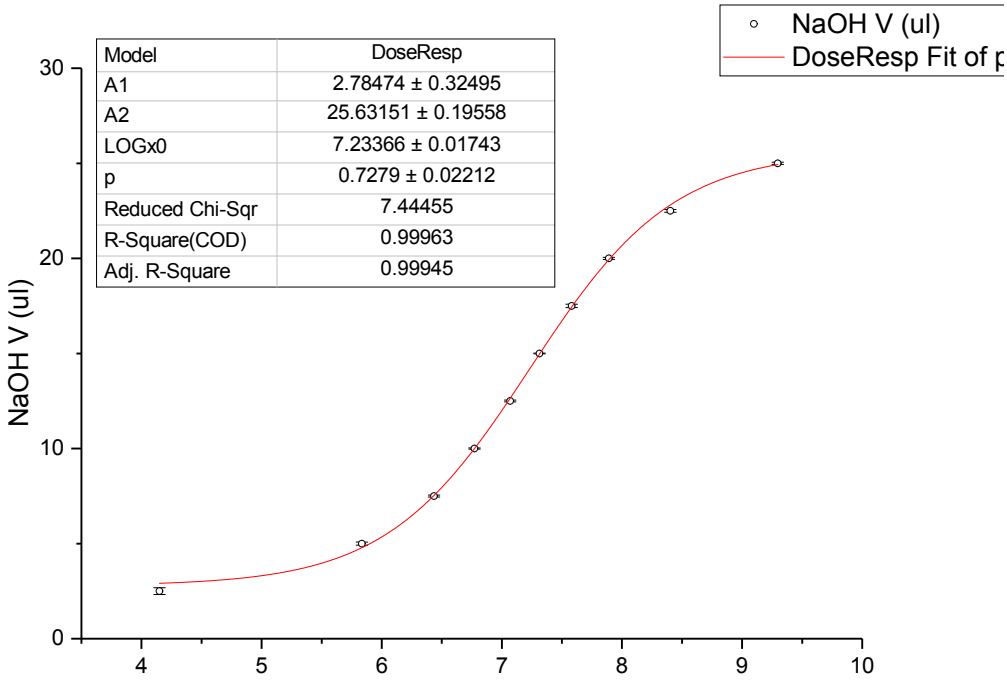
ESI-Figure 7. Hill plot analysis of compound **3**

6. pK_a determination

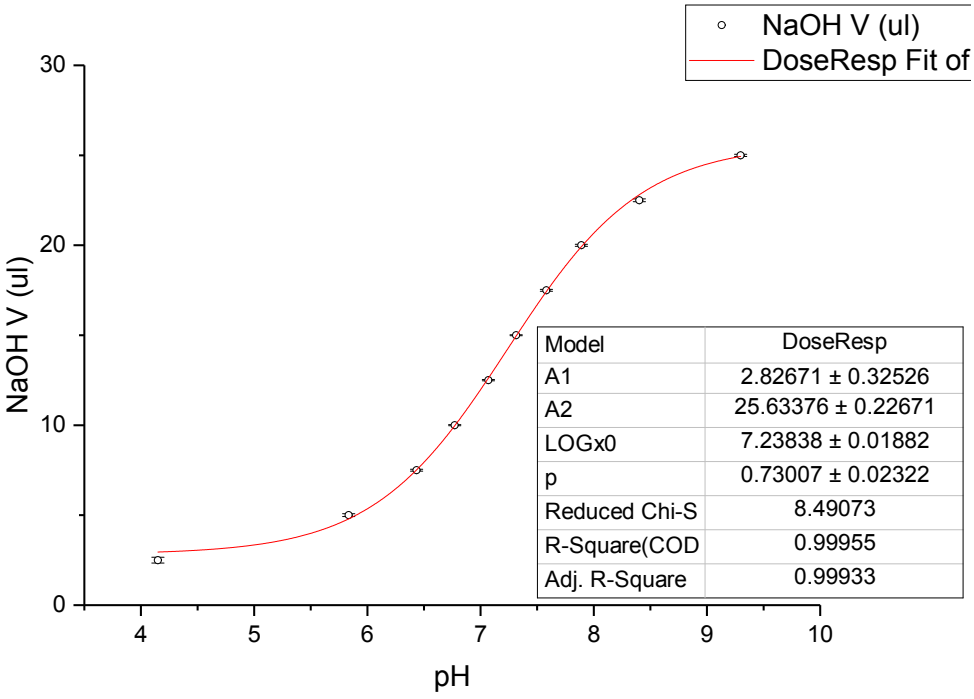
The pK_a values of compounds **1-3** were determined using a potentiometric experiment. A 1 mM solution in H_2O:DMSO (9:1) was prepared and acidified to pH 3 using 0.1 mM HCl. The solution was then titrated with a 0.1 mM NaOH solution, adding small aliquots of the base and registering the pH at each step. The pH variation was then plotted against the added volume of base and the data was fitted to a dose responsive sigmoidal fit using OriginPro 2017, where the obtained inflection point reflects the apparent pK_a value of the compounds.



ESI-Figure 8. Potentiometric p*K*a determination for compound **1.**



ESI-Figure 9. Potentiometric p*K*a determination for compound **2.**



ESI-Figure 10. Potentiometric p*K*a determination for compound **3**

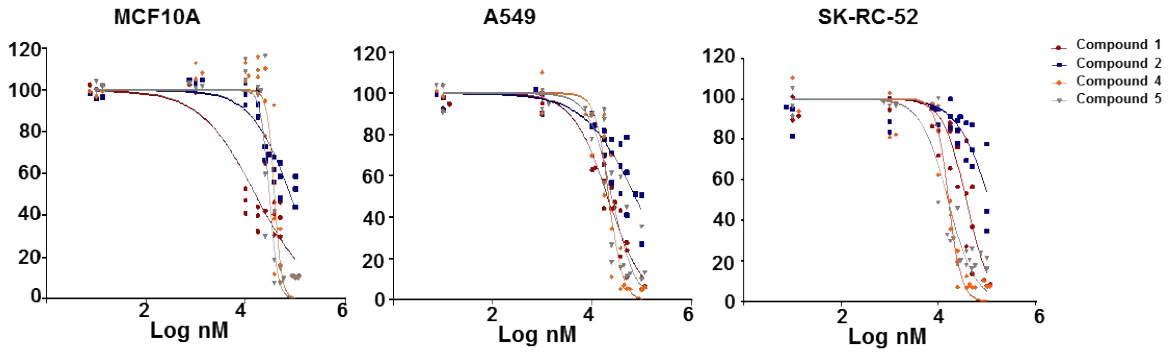
7. Cell assay

Cell culture

Five different cell lines were used for the in vitro studies, namely MCF10A (a non-tumorigenic epithelial cell line from breast), HEK293T (human embryonic kidney cell line), MCF7 (breast cancer cell line), A549 (lung cancer cell line) and SK-RC-52 (human renal carcinoma cell line). All the cells, except for MFC10A, were maintained in a humidified incubator at 37ºC under 5% CO_2 and grown using 1x D-MEM (Dulbecco's modified Eagle medium) with Sodium Pyruvate and without L-Glutamine (Invitrogen, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies), 1x MEM NEAA (Gibco, Life Technologies) ,1x GlutaMAX (Gibco, Life Technologies), 200 units/mL penicillin and 200 µg/mL streptomycin (Gibco, Life Technologies). In the case of MFC10A, the cells were cultured in DMEM:F12 media (with glutamine) supplemented with 5% horse serum (Life Technologies), 20 ng/ml EGF, 0.5 µg/ml Hydrocortisone, 100 ng/ml Cholera toxin all from Sigma-Aldrich, 10 µg/ml insulin (Life Technologies) and 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies).

Cell viability assay and IC50 calculation

10 000 cells/well were seeded in 96 well-plates and were treated with the compounds 24h after the seeding, to allow the cells to stabilize. The cells were incubated with 100µM, 50µM, 37,5µM, 25µM, 17,5µM, 10µM, 1µM and 0,01µM of the compounds for 48 hours. After this incubation period, the culture medium was removed and the cells were incubated with CellTiter-Blue (Promega) for 1h30min at 37ºC. The cells viability was evaluated by measuring the Emission Intensity in RFUs – relative fluorescent Units- with an Infinite M200 plate reader. IC50s were calculated with the help of GraphPad Prism5 software. All the results are shown as Mean + SEM of three independent experiments.



ESI-Figure 11. IC50 curves of compounds **1**, **2**, **4** and **5** determined after 48h incubation with MCF10A, A549 and SK-RC-52 cells.

Cell death mechanism evaluated through Annexin V/7AAD assay

100 000 A549 cells/well were seeded in 12-well plates and were allowed to stabilize for 24h before the assay. After the 24h period the cells were incubated with either 50 µM compounds for 1h or with IC50 values of the compounds for 48h incubation times. Immediately after the end of the incubation periods, the cells were detached, centrifuged at 1400rpm for 4min and re-suspended in 1mL of 1X PBS for a washing step (1400rpm, 4min), The cells were then re-suspended in 200µL of 1x Annexin-V binding buffer and centrifuged again (same conditions as before). Finally, the cells were re-suspended in a 2.5-100 dilution of Annexin-V in binding buffer and incubated at room temperature

for 15min, after which were washed with binding buffer and re-suspended in a 2-100 dilution of 7AAD in binding buffer. At this point the cells were immediately analyzed by Flow Cytometry, using a Fortessa X-20. The Annexin-V and 7AAD used in this assay were purchased from eBiosciences (88-8007-74, ANNEXIN V APOP DETECT KIT APC).

Notes and references

Disclaimer:
The content present herein reflects only the views of the authors and not the views of the University of Cambridge or the European Commission.

1 P. Timmerman, K. A. Jolliffe, M. Crego Calama, J.-L. Weidmann, L. J. Prins, F. Cardullo, B. H. M. Snellink-Ruël, R. H. Fokkens, N. M. M. Nibbering, S. Shinkai and D. N. Reinhoudt, *Chem. Eur. J.*, 2000, **6**, 4104–4115.
2 G. M. Sheldrick, *Acta Crystallogr. Sect. Found. Adv.*, 2015, **71**, 3–8.
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4 C. C. Overly, K. D. Lee, E. Berthiaume and P. J. Hollenbeck, *Proc. Natl. Acad. Sci.*, 1995, **92**, 3156–3160.