Supporting information for

Inhibition of NGLY1 inactivates the transcription factor Nrf1 and potentiates proteasome inhibitor cytotoxicity

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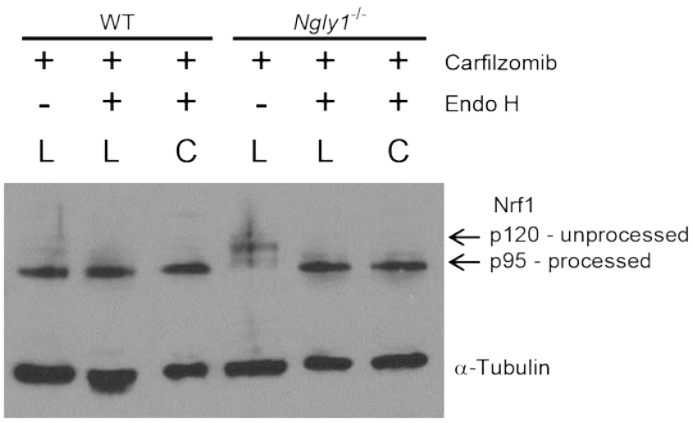


Figure S1. WT and *Ngly1*$^{-/-}$ MEFs were treated with the proteasome inhibitor carfilzomib (200 nM) for 12 h prior to harvest, cell lysis, denaturation, and treatment with Endo H (15000 U) for 16 h before immunoblotting as in Fig. 2B. L = whole-cell lysates, C = precipitated protein.

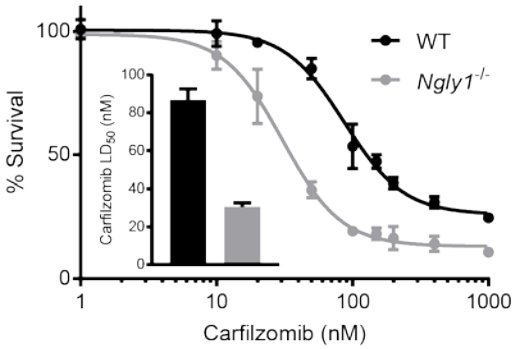


Figure S2. MEFs treated with carfilzomib in fresh media for 24 h then checked for survival compared to vehicle controls using the CellTiter-Glo 2.0 assay. Error bars are one standard deviation from the mean, n=3. Inset: The LD_{50} of carfilzomib in WT and Ngly1$^{-/-}$ MEFs, calculated by a 4-parameter variable slope fit. Error bars are standard error.

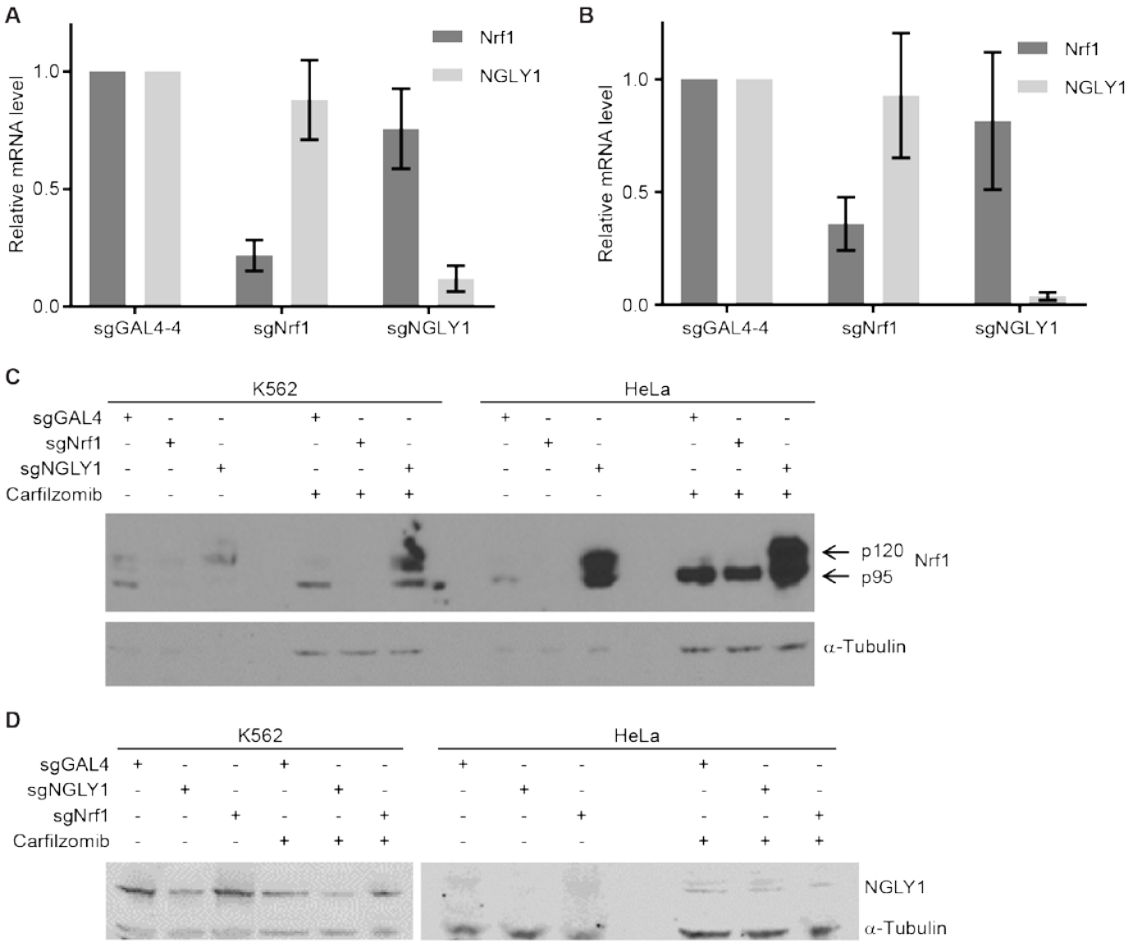


Figure S3. Transcript and protein levels of dCas9-KRAB knockdowns. Equal amounts of RNA from each CRISPRi K562 (**A**) and HeLa (**B**) cell lines was subjected to reversed transcription and quantitative PCR. The value of each target gene in each sample was first normalized internally by the value of GAPDH, and was then compared to that of the same gene in the sgGAL4-4 negative control cell line, which is set as 1. Shown are the mean ± S.D. (n=4). **C** and **D**) K562 and HeLa cells were treated with vehicle or 50 nM carfilzomib for 4 h before being harvested. The cell lysates were subjected to western blot for Nrf1 (**C**) or NGLY1 (**D**).

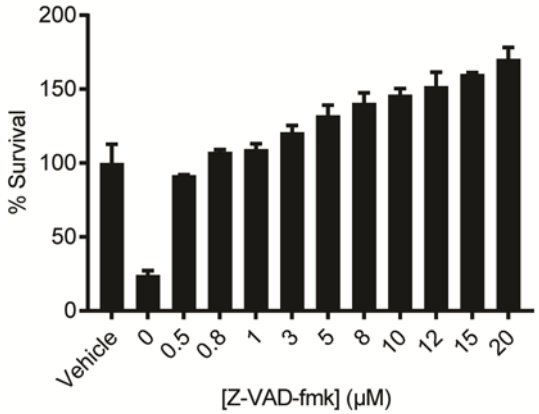
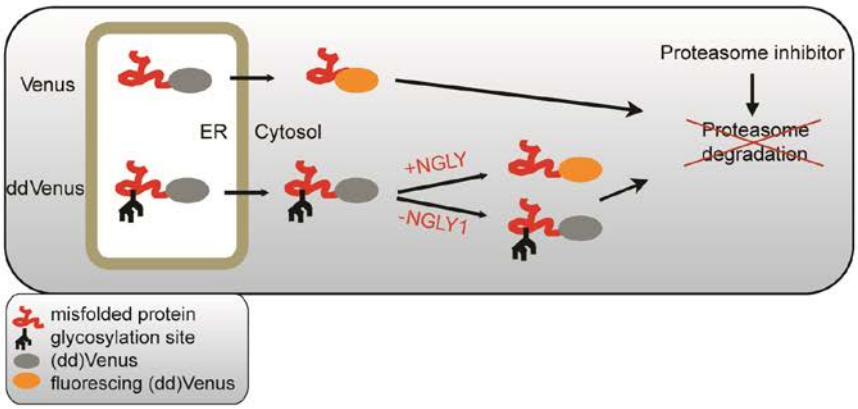
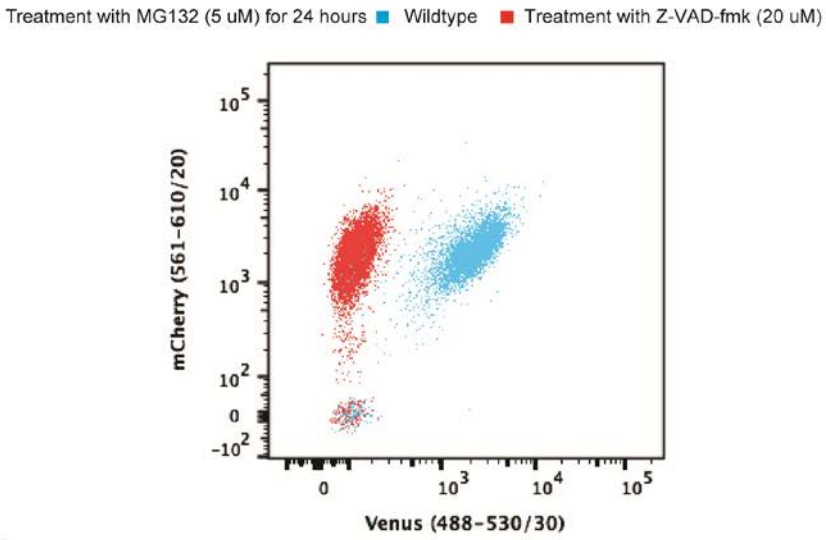


Figure S4. U266 cells were treated with carfilzomib (20 nM) or vehicle (DMSO) and Z-VAD-fmk for 24 h. Survival was calculated as percent luminescence of vehicle control using the CellTiter-Glo 2.0 assay, n=3. Error bars represent standard deviation.

A


Venus
ER
Cytosol
ddVenus
+NGLY
-NGLY1
Proteasome inhibitor
Proteasome degradation
misfolded protein
glycosylation site
(dd)Venus
fluorescing (dd)Venus

B


Treatment with MG132 (5 uM) for 24 hours
Wildtype
Treatment with Z-VAD-fmk (20 uM)
mCherry (561-610/20)
Venus (488-530/30)

C

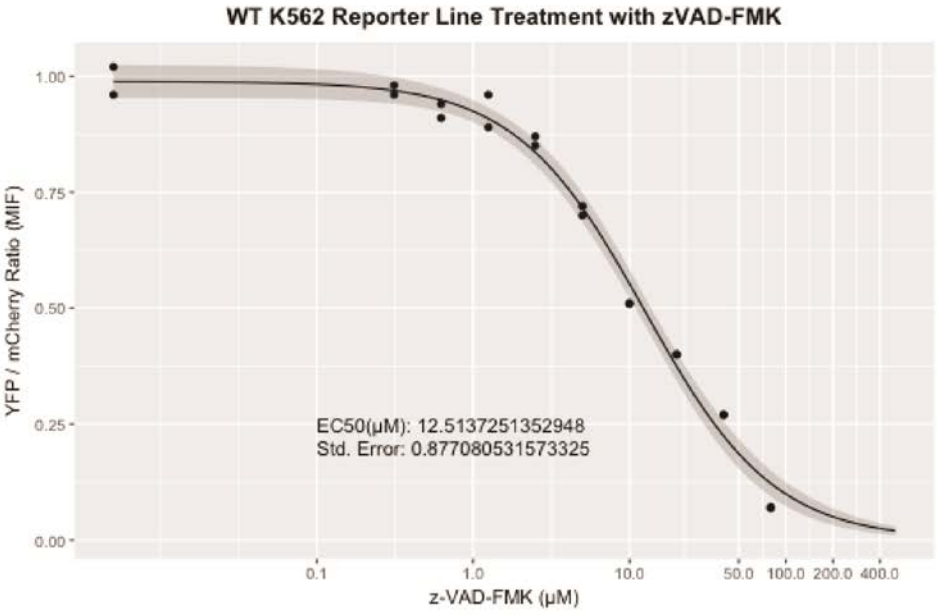

WT K562 Reporter Line Treatment with zVAD-FMK
YFP / mCherry Ratio (MIF)
z-VAD-FMK (μM)
EC50(μM): 12.5137251352948
Std. Error: 0.877080531573325

Figure S5. A) The ddVenus was cloned ahead of an IRES-mCherry in a lentiviral vector. This allows for expression control calculations as well as identification of reporter expressing cells. Treatment of the cells with a proteasome inhibitor is necessary for observation of fluorescence. The accumulation of Venus signal corrected by the mCherry signal (Venus to mCherry fluorescence ratio) is used to determine the amount of deglycosylation occurring in the cells. **B)** K562 cells were infected with ddVenus/mCherry lentivirus and selected by mCherry fluorescence. Treatment of the cells with MG132 (5 μM) or carfilzomib (1 μM) for 6 h resulted in accumulation of Venus signal (as shown in blue). Treatment of the cells with Z-VAD-fmk (20 μM) for 24 hours before proteasome inhibition resulted in a ~2.5 fold reduction in Venus signal (as shown in red), suggesting that NGLY1 inhibition is responsible for ddVenus deglycosylation. **C)** Titration of Z-VAD-fmk was carried out to determine the effective range of the ddVenus reporter. Similar to **B**, K562 cells were treated with a range of NGLY1 inhibitor for 24 hours, then proteasome inhibition was carried out for 6 hours, and finally these treatments were followed by FACS analysis. Fluorescence intensity ratios were used to calculate the dose response curve to determine the effective range for detection in the K562 cell line.

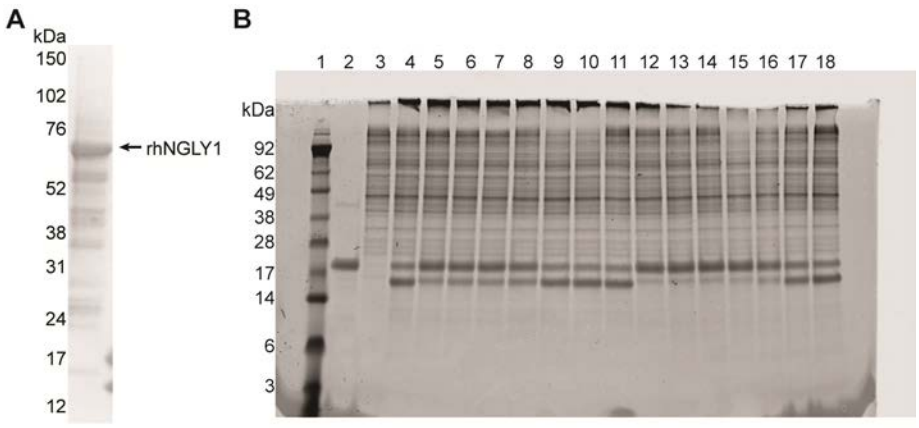


Figure S6. Purified rhNGLY1 and RNase B deglycosylation assay. **A)** rhNGLY1 (10 µg) was mixed with 4X Laemmli Sample Buffer containing 10% β-Me and separated on a 4-20% SDS-PAGE gel. **B)** RNAse B deglycosylation by NGLY1, full gel from Figure 7A. This gel is not representative of purity of NGLY1 sample due to noted aggregation of NGLY1 upon heating the protein sample in high salt concentrations before separation by SDS-PAGE. RNase B is represented by the lower two bands. Upper band: glycosylated. Lower band: deglycosylated. Lanes: 1) Ladder; 2) RNase B with no NGLY1; 3) NGLY1 with no RNase B; 4) Reaction with no inhibitor; 5-11) Reaction with WRR139 (100, 50, 25, 10, 1, 0.1, and 0.01 µM, respectively); 12-18) Reaction with Z-VAD-fmk (100, 50, 25, 10, 1, 0.1, and 0.01 µM, respectively).

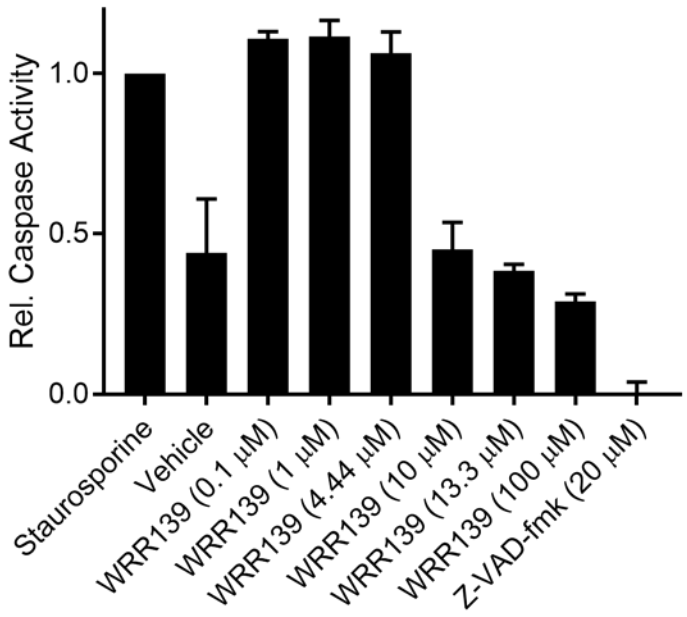


Figure S7. Inhibition of caspase activity by WRR139 or Z-VAD-fmk. U266 cells were treated with vehicle (DMSO) or staurosporine (1 μM) at a density of 10^6 cells/mL. The staurosporine-treated cells were further treated with WRR139 or Z-VAD-fmk. The cells were incubated for 8 hours and then treated with Caspase-Glo reagent and luminescence was measured on a plate reader. Caspase activity was normalized to cells treated with only Staurosporine. Staurosporine = cells treated with staurosporine (1 μM) and no inhibitor. Vehicle = cells treated only with DMSO. All other lanes = cells treated with indicated inhibitor and staurosporine (1 μM). Error bars represent standard deviation.

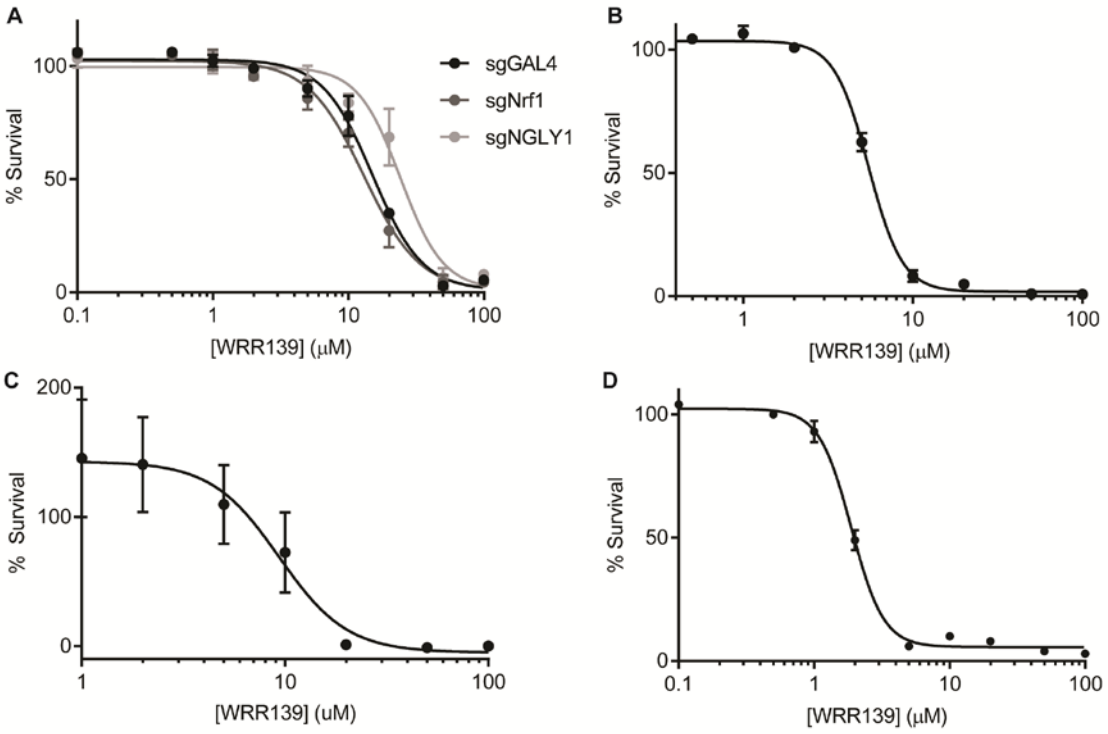


Figure S8. Toxicity of WRR139 in HeLa (**A**), U266 (**B**), NCI-H929 (**C**), and Jurkat (**D**) cell lines. Cells were treated with WRR139 for 24 h and measured for survival with the CellTiter-Glo 2.0 assay and compared to vehicle control. N = 3, error bars are 1 standard deviation from the mean.

Materials and methods

Reagent and Synthesis Instrumentation

Unless otherwise stated commercial reagents were used as received without further purification. NMR spectra were recorded on a Varian 400 MHz spectrometer and were adjusted to deuterated solvent ($CDCl_3$: ^{1}H NMR = 7.26, ^{13}C NMR = 77.16). Multiplicities in NMR spectra were denoted by the following abbreviation: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. High resolution mass spectra (HRMS) were obtained on Bruker micrOTOF-Q II mass spectrometer using ESI-TOF (electrospray ionization-time of flight).

Cell culture

Primary MEFs derived from knockout animals and immortalized using SV40 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) at 37 °C in 5% CO_2.

Nrf-3xFLAG overexpressing HEK293 cells (RDB-2867 were obtained by cloning Human Nrf1 coding region along with a C-terminal 3×Flag sequence into pcDNA3.1+ (Invitrogen, Carlsbad, CA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) at 37 °C in 5% CO_2.

K562 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) at 37 °C in 5% CO_2.

U266-B1 cells were grown in RPMI-1640 supplemented with 15% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) at 37 °C in 5% CO_2.

NCI-H929 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) and 50 mM beta-mercaptoethanol (BME) at 37 °C in 5% CO_2.

Jurkat cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) at 37 °C in 5% CO_2.

For dCas9-KRAB expressing cell lines, HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) at 37 °C in 5% CO_2. K562 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (Clontech Laboratories), penicillin, and streptomycin (Fisher Scientific) at 37 °C in 5% CO_2.

Immunoblot Analysis

Cells were lysed in RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% NP40,1% Na, Deoxycholate, 0.1% SDS, and 1 mM EDTA) supplemented with protease and phosphatase inhibitor cocktail (Pierce). For detecting Nrf1, a rabbit monoclonal antibody raised against an epitope surrounding Gly129 of human TCF11 (D5B10, Cell Signaling) was used. For detecting

NGLY1 a rabbit polyclonal antibody (HPA036825, Sigma Aldrich) was used. Other immunoblots were performed with antibodies specific for FLAG tag (A8592, Sigma-Aldrich), α-Tubulin (2144, Cell Signaling) and β-actin (13E5, Cell Signaling). Blots were developed by either using an HRP secondary antibody (anti-rabbit IgG, HAF008; R&D Systems), a chemiluminescent substrate (34080 SuperSignal West Pico, Thermo Scientific) and a film developer (Optimax X-Ray Film Processor, PROTEC GmbH & Co. KG, Germany) or by using infrared-based secondary antibodies (800 CW Goat anti-Rabbit, 926-32211; Li-COR Biosciences, USA) and a digital Fluorescence Image system (ODYSSEY CLx; Li-COR Biosciences, USA).

Endo H treatment of MEF cell lysates

WT and *Ngly1*$^{-/-}$ MEFs were grown to 70% confluence and treated with vehicle or carfilzomib (200 nM, 12 h). Cells were lysed and the protein levels were quantified using a BCA assay (23227 Pierce BCA Protein Assay Kit, Thermo Scientific). Quantification was performed in either whole cell lysates (lysate buffer) or proteins were precipitated with ice-cold acetone and resuspended in denaturing buffer. Whole-cell lysates containing 20 µg protein or 20 µg of precipitated protein (resuspended in denaturing buffer) were dissolved in denaturing buffer containing 0.5% SDS 40 mM DTT (provided with the P0703L Endo H_f kit from New England BioLabs) for 10 min at 100 °C. After addition of Glycobuffer 3 (total volume 30 µL) samples were treated with Endo H_f (15,000 U) and incubated at 37 °C over night. Lysates of control samples were prepared the same way but not treated with Endo H_f. Proteins were separated by SDS-PAGE and Nrf1 levels were visualized by immunoblot analysis.

Plasmid construction and rescue transfections of NGLY1 in *Ngly1*$^{-/-}$ MEFs

NGLY1 cDNA was obtained from the NIH Mammalian Gene Collection (Reference: http://genome.cshlp.org/content/19/12/2324.long). A catalytic dead version (Cyesteine 309 to Serine, C309S) was generated by PCR-based primer extension approach using the following primers:

fw:
GGAAACAAGATGTGGACGGTCTGGCGAGTGGGCCAATTG
rv:
CAATTGGCCCACTCGCCAGACCGTCCACATCTTGTTTCC

Each cDNA was PCR amplified with InFusion_NGLY1_F and InFusion_NGLY1_R primers to add homologous regions to the pLEX vector to the ends of each cDNA. pLEX destination vector was created by double-restriction digestion. 1 µg of uncut pLEX vector was digest with 1 µL BamHI (NEB) and 1 µL XhoI (NEB) with 2 µL 10x CutSmart Buffer (NEB) in 20 µL for 1 hour at 37 °C. After PCR amplification of cDNA and restriction digestion of the pLEX vector, products were gel purified using a 1% agarose gel. DNA was extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research). 100 ng of purified cut pLEX vector was mixed with 200 ng of either WT or Mut NGLY1 PCR DNA with 1 µL of 5x InFusion HD Enzyme Premix. Samples were incubated for 15 minutes at 50 °C and then placed on ice. 1 µL of each InFusion reaction was used to transform Top10 Competent *e. coli* cells (Thermo Fischer Scientific) by heat shocking at 42 °C for 30 seconds. Individual colonies were selected on ampicillin resistant agar plates and plasmid minipreps were generated using Zyppy Plasmid Miniprep Kit (Zymo Research).

For transfection experiments, NGLY1 WT or *Ngly1*$^{-/-}$ MEFs were seeded in 6-well plates at 100,000 cells per well. One day after seeding, fresh media was added to all wells. For each well, 2 µg of pLEX-EV (empty vector), pLEX-NGly1-WT, or pLEX-NGly1-Mut plasmid DNA was combined with 250 µL Opti-MEM (Thermo Fisher Scientific) and separately 10 µL of Lipofectamine 2000 (Thermo Fisher Scientific) was combined with 250 µL Opti-MEM. Each reaction was incubated at 25 °C for 5 minutes after which they were combined with a final volume of ~510 µL. DNA-Lipofectamine mixes were gently pipetted for mixing and incubated at 25 °C for 15 minutes. After this incubation, the ~510 µL of each mix was added, drop-wise, to each well of the previously plated MEF cells. Media was changed 16 hours after transfection to normal growth media. Finally, cells were treated with carfilzomib by changing media on each well to 2 mL of 50 nM carfilzomib. After 4 hours of treatment, cells were rinsed once in 1x PBS, PBS aspirated, and cells were lysed in Lysis Buffer (50 mM HEPES, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 0.2% Triton X-100, 0.5% N-lauroylsarcosine) and prepared for western blot analysis.

InFusion_NGLY1_F
CTCTACTAGAGGATCCATGGCGGCGGCGGCATTGGG
InFusion_NGLY1_R
GCCCTCTAGACTCGAGCTAAAGGTCACTGAATTTTATAA

Luciferase Assays

The construct 3xPSMA4-ARE-Luc (RDB-2415 was described previously.[1] Briefly, an oligo (5'-cgagccgtgggcacga TGACTCTGCA ccgcctcctctgagccgtgggcacga TGACTCTGCA ccgcctcctctgagccgtgggcacga TGACTCTGCA ccgcctcctctg-3') containing three copies of a putative ARE (shown in uppercase) derived from the first intron of the human PSMA4 gene was annealed to its corresponding reverse-complement oligo and cloned into pGL3-promoter vector (Promega). The promoter reporter was generated by inserting this construct upstream of a minimal SV40 promoter, driving the expression of a firefly luciferase.

Cells were transiently transfected with the *firefly* (promoter reporters) and *renilla* luciferase (pRL-TK; Promega) constructs along with effector plasmid as required. Cells were treated with with compounds or vehicle control for 12 hours. Afterwards cells were lysed and the luciferase assays were performed using the dual luciferase reporter assay system (E1910; Promega) according to the instructions from the manufacturer. The firefly luciferase activity was normalized to *renilla* luciferase activity for all experiments.

Quantitative reverse transcription PCR

RNA from mouse embryonic fibroblasts was isolated using the RNeasy kit (Qiagen) and cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad). With this cDNA as a template, quantitative PCR (qPCR) was performed using iTaq universal SYBR green mix (Bio-Rad) as per manufacturer's recommendations. The primers used for qPCR are as follows: PSMA7 (forward: 5'-AAC GTC TGT ATG GCC TTT GC-3'; reverse: 5'-CTC ACT GGG TCC TCC ACT GT-3'), PSMB7 (forward: 5'-CTG TCT TGG AAG CGG ATT TC-3'; reverse: 5'- GCA ACA ACC ATC CCT TCA GT -3'), PSMC4 (forward: 5'- TGG TCA TCG GTC AGT TCT TG -3'; reverse: 5'-CGG TCG ATG GTA CTC AGG AT-3'), PSMD12 (forward: 5'-TCA CAG ACC TGC CAG TCA AG-3'; reverse: 5'-AGG TTT TAG TCA GCC GAG CA-3'), and GAPDH (forward: 5'-AAC TTT GGC ATT GTG GAA GG-3'; reverse: 5'-GGA TGC AGG GAT GAT GTT CT-3').

Immunofluorescence followed by confocal imaging

MEFs and Nrf1-3xFLAG overexpressing HEK293 cells were grown to 80% confluency on cover slips. Cells were treated with NGLY1, caspase, and proteasome inhibitors as indicated and allowed to recover in fresh media for one to two hours before cells were fixed using 100% ice cold methanol. Cells were treated with a primary rabbit polyclonal antibody raised against the middle region of Nrf1 (SC-13031, Santa Cruz) and a secondary polyclonal Alexa Fluor®647 AffiniPure Fab Fragment Goat Anti-Rabbit IgG (H+L) (Jackson Immuno Research). For staining the endoplasmic reticulum, cells were treated with a primary chicken polyclonal antibody raised against Calnexin (Ab140818, Abcam) followed by a secondary polyclonal Alexa Fluor®594 AffiniPure Fab Fragment Goat Anti-Chicken IgY†† (IgG) (H+L) (Jackson Immuno Research). After washing with PBS cover slips were treated with ProLong®Diamond Antifade Mountant (P3697, Thermo Fischer) containing 49,6-Diamidino-2-Phenylindole (DAPI) for nuclear DNA staining, placed on slides and dried overnight. Thereafter, subcellular location of Nrf1 (far-red channel) was examined by confocal imaging using a Nikon Eclipse Ti and a 60x magnification. Images were analyzed using Imaris analysis software, version 8.4.0. Bar =10 mm. Original images available upon request.

Cell survival experiments with CellTiter-Glo 2.0

Cell survival experiments using the CellTiter-Glo 2.0 (G9242, Promega) assay were performed in opaque white flat-bottomed 96-well plates. The cells (1.0-2.0 x 10^4 in 100 µL fresh media) were treated under the desired conditions and incubated for 24 h before addition of 100 µL assay reagent. Viability was calculated as percent signal compared to vehicle treated cells. Non-linear regression analysis and IC_{50} calculations were performed with GraphPad Prism 7.0. Read-out was performed by measuring the luminescence using a Spectramax®i3x (Molecular Devices) plate reader.

MEF Viability Assays

Cells were treated in 96-well plates with carfilzomib in concentrations ranging from 1 to 1000 nM for 24 h. After the incubation, the plate was retrieved and treated with the CellTiter-Glo 2.0 assay. After 10 minutes, the plate was read for luminescence using a Spectramax®i3x (Molecular Devices) plate reader.

Generation of CRISPRi-mediated knockdown K562 cell lines:

The CRISPRi K562 and HeLa cell lines that stably express dCas9-KRAB and the third-generation lentiviral system were obtained from the group of Jonathan Weissman (University of California, San Francisco).[2] The generation of CRISPRi-based knockdown K562 cell lines was carried out according to the published procedure.[2] First, protospacer sequences with the highest predicted CRISPRi activity scores reported by Horlbeck *et al.*[3] were cloned into a pU6-driven locus in the lentiviral transfer plasmid.[2] An sgGAL4-4 was used as negative control, as previously described.[4] The lentivirus was produced in HEK293T cells using a third-generation lentiviral plasmid system.

For lentiviral infection, cells were first seeded at a density of 0.1 million/ml in a 24-well plate overnight. The next day, lentivirus carrying individual sgRNA vector was added to each well, and the cells were then returned to normal cell culture incubation condition. Two days after infection, puromycin was added to a final concentration of 1.25 µg/ml, and the selection was

continued for 4 days. All experiments performed on the sgRNA-transduced cells were then carried out with no more than ten passages after the puromycin selection.

To determine the level of gene knockdown, 0.2 million cells were collected, and RNA was extracted by the Quick-RNA™ MicroPrep kit (Zymo Research), according to the manufacturer's instruction. 200 ng of the extracted RNA was reversed transcribed to cDNA using the ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs) with the supplied Oligo d(T)23 VN primer, according to the manufacturer's instruction. Quantitative PCR reactions were prepared using the SYBR® Green PCR Master Mix (Thermo Fisher Scientific), according to the manufacturer's instruction. PCR reactions were carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).

Sequence of protospacers of each sgRNA construct:
NFE2L1 (Nrf1): 5'-GAAGCTCCGGCGCCGAGAGT-3'
NGLY1: 5'-GTGAGGCGCCTGCTCAGTGT-3'
GAL4-4: 5'-GAACGACTAGTTAGGCGTGTA-3'

Primer sequences for quantitative PCR:
GAPDH:
FW: 5'-GGTGGTCTCCTCTGACTTCAACA-3'
RV: 5'-GTTGCTGTAGCCAAATTCGTTGT3'

NFE2L1:
FW: 5'- AGTGGCAAGATCTCATGTCC-3'
RV: 5'- GCTGAAGAGTAAGAAGTCCTGG-3'

NGLY1:
FW: 5'-AATATCTGGGTCAGTGGCTTG-3'
RV: 5'- CATTTTCCACACGCCATTCTC-3'

Carfilzomib sensitivity of CRISPRi-knockdown K562 cell lines
A master solution of carfilzomib was made in DMSO at a concentration of 10 mM and was used freshly or stored at -80°C without repeated freeze-thaw cycles. Cells were seeded at a density of 0.2 million cells/ml in 100 µl of medium in each well of a 96-well plate for 18 hours before treatment. The next day, serial-diluted Carfilzomib was added to the cells with a final concentration of DMSO of 0.1% (v/v) in each well. For K562 cells, after 48 hours of incubation at 37°C with 5% CO_2, the plate was retrieved and stained with the LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific), according to the manufacturer's instruction. The cells were then counted on an Accuri C6 flow cytometer (BD Biosciences, CA, USA). A total of 10,000 events were collected for each well. The % survival was calculated by dividing the percent of calcein-AM positive cells in each well with that of the DMSO-treated vehicle control. For HeLa cells, after 48 hours of incubation at 37°C with 5% CO_2, the plate was retrieved and stained with the CellTiter-Glo 2.0 assay (Promega), according to the manufacturer's instruction. The luminescence was measured on a Spectramax®i3x plate reader (Molecular Devices). The %

survival was calculated by dividing the total luminescence in each well with that of the DMSO-treated vehicle control.

Development of Cresswell Assay in K562:

SS-C-Venus and SS-C-ddVenus reporter sequences (Grotzke, J.E., Lu, Q., et al. 2013) were introduced into a modified pRetro-IRES vector (Clontech), expressing mCherry driven by the IRES. The pRetroX-IRES-mCherry was created by amplifying the mCherry sequence from pCMV-mCherry-C3 (Clontech) using PJ221 forward primer and PJ222 reverse primer. The PCR product was cut with XhoI and BmgBI enzymes and replaced the DsRed sequence which was cut from pRetroX-IRES-dsRed using XhoI enzyme and BmgBI enzyme.

The SS-C-Venus sequence was amplified with the primer PJ181 and PJ219 from plasmid pcDNA-SS-C-Venus (Grotzke, J.E., Lu, Q., et al. 2013), SS-C-ddVenus sequence was amplified with the primer PJ181 and PJ227 from plasmid pcDNA-SS-C-ddVenus (Grotzke, J.E., Lu, Q., et al. 2013). Both reporter sequences were cloned into the MCS with Not1 and BamH1 to give rise to pRetro-IRES-mCherry C3-SS-C-Venus and pRetro-IRES-mCherry C3-SS-C-ddVenus.

PJ221	ACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGGTGAGCAAGGGCGAGGAGG
PJ222	CTTTTATTTTATCCTCGAGCATTCTAAGCTCGTCCATGCCGCCG
PJ181	CCAGTGTGGTGGAATTCTGCAGATATCCAGC
PJ227	GGCATCGCCCTCtcctaccggtGGATCCCGGGTTTAAACGGGCCCCC
PJ219	ccggtaggccGGATCCGCTGATCAGCGGGTTTAAACGGGCCCC

These plasmids were used to stably integrate the SS-C-Venus and SS-C-ddVenus reporter in the K562 WT cell line by viral transduction according to Clontech's manual. Positive transduced cells were sorted by mCherry fluorescence.

Prior to analysis, cells were washed twice with PBS + 2% FCS, resuspended in the same, strained through a 40-µm filter, and incubated with 0.5 µg ml DAPI for live/dead detection on ice until analysis. FACS analysis was carried out on a BD LSR Fortessa measuring Venus fluorescence with 50 mW 488 nm excitation paired with a 530/30 nm band pass filter; mCherry fluorescence was measured with 75 mW 561 nm excitation paired with a 610/20nm band pass filter. Live single cells were selected by exclusion of DAPI positive cells utilizing 20 mW 355 nm excitation paired with a 450/50 band pass filter. The geometric mean of the Venus and mCherry signal was used to compute the Venus/mCherry ratio. Analysis was carried out using FlowJo 10.1r7 software, Treestar. Raw .fcs data files and analysis are available upon request.

Screen of 553 compounds with the Cresswell Assay

Library of cysteine protease inhibitor-like compounds supplied by Prof. Matthew Bogyo at Stanford University. A full list of compounds screened can be obtained upon request.

96-well plates were each prepared with a negative control (2 µL DMSO), a positive control (1 µL DMSO, 1 µL 100 µM carfilzomib in DMSO), an inhibition control (1 µL 100 µM carfilzomib in

DMSO and 1 µL 10 mM Z-VAD-fmk in DMSO), and test wells (1 µL 100 µM carfilzomib in DMSO and 1 µL 1 mM library compound in DMSO). In fresh culture media, ddVenus-expressing K562 cells were suspended at 1 million cells/mL and plated at a volume of 100 µL per well in the prepared plates. Final concentrations were 1 µM carfilzomib, 100 µM Z-VAD-fmk, 10 µM library compound, and 2% DMSO. Plates were incubated for 6 hours at 37 °C with 5% CO_2. Following incubation samples mean fluorescence in FL1 was measured by automated flow cytometry on an Accuri C6 Flow Cytometer (BD Biosciences, CA, USA), with cutoffs at 50 µL and 50,000 events. Hits were determined to be any compound that decreased fluorescence below 3 standard deviations below the mean of the positive control (maximum fluorescence). Z-factor for the assay ranged between 0.38-0.85.

Generation and purification of recombinant human NGLY1

Recombinant human NGLY1 (rhNGLY1) was expressed in *Spodoptera frugiperda* (Sf9) insect cells using the Bac-to-Bac TOPO Expression System (Invitrogen, Carlsbad, CA). Briefly, the DNA sequence of human NGLY1, with a C-terminal TEV cleavage site and 6xHis epitope tag, codon-optimized for expression in insect cells, was synthesized and subcloned into the mammalian vector pD2610-v14. This gene synthesis step was performed by ATUM (formerly DNA2.0, Menlo Park, CA). The NGLY1 sequence was PCR-amplified using the primers
5'-ATGGCCGCCGCTGCTCTTGGTTCTTCTTCCGGTT-3' and
5'-CAGGTCAGAGAACTTAATAATAATTTCCAGAC-3',
and cloned into the pFastBac/CT-TOPO vector, upstream of the TEV cleavage site and 6xHis tag, following the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Recombinant bacmids were generated by transforming DH10Bac cells with pFastBac/CT-TOPO containing the NGLY1 sequence, and bacmids were isolated. Sequences were verified at all stages of the cloning process (Genewiz, South Plainfield, NJ). To generate baculovirus, Sf9 cells were transfected with recombinant bacmid using Cellfectin transfection reagent (Invitrogen, Carlsbad, CA). The virus went through two further rounds of amplification to generate high titer baculovirus stock. To express rhNGLY1, Sf9 cells were infected with baculovirus at an MOI of 15-30 and grown for 72 hr at 27°C. Cells were collected and washed with PBS before storage at -80 °C.

Purification steps were performed at 4 °C. Cells were resuspended in 50 mM sodium phosphate pH 7.4, 500 mM NaCl, 20 mM β-Mercaptoethanol, 250 U/ml Pierce™ Universal Nuclease for Cell Lysis (Thermo Fisher Scientific, Waltham MA), Pierce™ EDTA-free protease inhibitor (Thermo Fisher Scientific) and then lysed by homogenization. The lysate was centrifuged at 20,000 g for 20 mins. The soluble extract was passed through a 0.45 µm vacuum filter and then applied at 5 ml/min to a 5 ml Ni Sepharose High Performance column (GE Healthcare Life Sciences, Pittsburgh PA) equilibrated with column buffer (50 mM sodium phosphate pH 7.4, 500 mM NaCl, 20 mM β -Mercaptoethanol) using the AKTA avant 25 chromatography system (GE healthcare). rhNGLY1 protein was eluted with column buffer containing 500 mM imidazole. The eluate was then applied at 2.0 ml/min to a Sephacryl S-200 HR column (GE Product code: 17119501) equilibrated with column buffer. The fractions containing rhNGLY1 were collected and concentrated using an Amicon Ultra-15 30K Centrifugal Filter device. β -Mercaptoethanol was subsequently removed using a PD MiniTrap G-25 column (GE Healthcare) and 1X Halt™

Protease Inhibitor EDTA-free (Thermo Fisher) was added to the rhNGLY1 which was stored at 4 °C.

In vitro RNase B deglycosylation assay

RNase B (1 mg/mL) was reduced with 5 mM DTT in the presence of 6 M guanidine hydrochloride at 42 °C for 1 hour. The RNase B was diluted 2-fold in doubly distilled water, and iodoacetamide was added to a final concentration of 25 mM. The solution was kept in the dark at room temperature for 1 hour. The protein was buffer exchanged into PBS using a PD Minitrap column (GE Healthcare Life Sciences). The protein was stored at -20 °C for later use.

The in vitro activity of rhNGLY1 was observed by setting up the following reaction:
rhNGLY1 stock (5 µL, 750 µg/mL) solution in buffer (50 mM phosphate, 500 mM NaCl, pH 7.4) was placed in a PCR tube. To this, 5 µL buffer followed by 1 µL of inhibitor (vehicle, WRR139, or Z-VAD-fmk) diluted in DMSO were added. This mixture was incubated at 37 °C for 1 hour before addition of 10 uL of 10 µM RNase B prepared as described above. The resulting solution was incubated at 37 °C for 1 hour. At this time, the reaction was quenched by the addition of 7 µL 4x SDS and the samples were heated at 90 °C for 10 minutes before being run on SDS-PAGE in an 18-well Criterion 12% Bis-Tris Pre-cast gel (180V, 40 min). The gel was visualized with Aquastain (Bulldog Bio).

Caspase 3/7 inhibition assay:

1 million U266 cells were suspended in 10 mL RPMI 15% BSA +PS, and plated into a flat-bottom, white-walled 96-well plate with 100 µL per well. The plate was incubated at 37 °C overnight. The following day, to the cells was added staurosporine (apoptosis inducer, final concentration 1 µM) or vehicle (no induced apoptosis), WRR139 or Z-VAD-fmk or DMSO to a final concentration of 2% DMSO (v/v). The plate was incubated for 8 hours at 37 °C. At this time, the Caspase-Glo 3/7 (Promega) kit reagent was prepared as described in the protocol, and after equilibration to 23 °C the reagent (100 µL) was added to each well. The plate was allowed to incubate at room temperature for 60 minutes before being measured for luminescence on a Spectramax i3x (Molecular Devices) plate reader. Caspase inhibition was calculated by subtracting background signal from media containing no cells, then taking the percent signal compared to the cells with apoptosis induced (staurosporine only).

Cytotoxicity of WRR139:

U266, H929: U266, H929, cells were seeded at a density of 0.1 million cells/mL in complete RPMI medium 100 µL per well in an opaque white 96-well plate. The following day WRR139 was added in DMSO to final concentrations as shown for a maximum final concentration of 1% DMSO (v/v). After 24 h of incubation at 37 °C with 5% CO_2, the plate was retrieved and treated with the CellTiter-Glo 2.0 assay. After 10 minutes, the plate was read for luminescence using a Spectramax®i3x (Molecular Devices) plate reader.

Jurkat, HeLa: Jurkat cells were seeded at a density of 0.1 million cells/mL in complete RPMI medium 100 µL per well in an opaque white 96-well plate. The following day WRR139 was added in DMSO to final concentrations as shown for a maximum final concentration of 0.1% DMSO (v/v). After 24 h of incubation at 37 ºC with 5% CO_2, the plate was retrieved and treated with the CellTiter-Glo 2.0 assay. After 10 minutes, the plate was read for luminescence using a Spectramax®i3x (Molecular Devices) plate reader.

Cell survival with carfilzomib and NGLY1 inhibitors:

U266, H929: U266, H929, cells were seeded at a density of 0.1 million cells/mL in complete RPMI medium 100 µL per well in an opaque white 96-well plate. The following day, carfilzomib, DMSO, and WRR139, or Z-VAD-fmk were added in DMSO to final concentrations as shown for a maximum final concentration of 1% DMSO (v/v). After 24 h of incubation at 37 °C with 5% CO_2, the plate was retrieved and treated with the CellTiter-Glo 2.0 assay.

Jurkat: Jurkat cells were seeded at a density of 0.1 million cells/mL in complete RPMI medium 100 µL per well in an opaque white 96-well plate. The following day, carfilzomib, DMSO, or WRR139 were added in DMSO to final concentrations as shown for a maximum final concentration of 0.2% DMSO (v/v). After 24 h of incubation at 37 °C with 5% CO_2, the plate was retrieved and treated with the CellTiter-Glo 2.0 assay.

K562 dCas9: Cells were seeded at a density of 0.1 million cells/mL in complete RPMI medium 100 µL per well in an opaque white 96-well plate. The following day, carfilzomib, DMSO, or WRR139 were added in DMSO to final concentrations as shown for a maximum final concentration of 0.2% DMSO (v/v). After 24 h of incubation at 37 °C with 5% CO_2, the plate was retrieved and treated with the CellTiter-Glo 2.0 assay.

References:

(1) Radhakrishnan, S. K.; Lee, C. S.; Young, P.; Beskow, A.; Chan, J. Y.; Deshaies, R. J. Transcription Factor Nrf1 Mediates the Proteasome Recovery Pathway after Proteasome Inhibition in Mammalian Cells. *Mol. Cell* **2010**, *38* (1), 17–28.

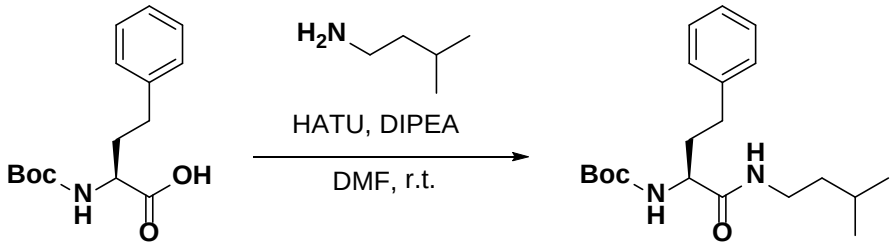
(2) Gilbert, L. A.; Horlbeck, M. A.; Adamson, B.; Villalta, J. E.; Chen, Y.; Whitehead, E. H.; Guimaraes, C.; Panning, B.; Ploegh, H. L.; Bassik, M. C.; et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **2014**, *159* (3), 647–661.

(3) Horlbeck, M. A.; Gilbert, L. A.; Villalta, J. E.; Adamson, B.; Pak, R. A.; Chen, Y.; Fields, A. P.; Park, C. Y.; Corn, J. E.; Kampmann, M.; et al. Compact and Highly Active next-Generation Libraries for CRISPR-Mediated Gene Repression and Activation. *Elife* **2016**, *5*, e19760.

(4) Gilbert, L. A.; Larson, M. H.; Morsut, L.; Liu, Z.; Brar, G. A.; Torres, S. E.; Stern-Ginossar, N.; Brandman, O.; Whitehead, E. H.; Doudna, J. A.; et al. CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes. *Cell* **2013**, *154* (2), 442–451.

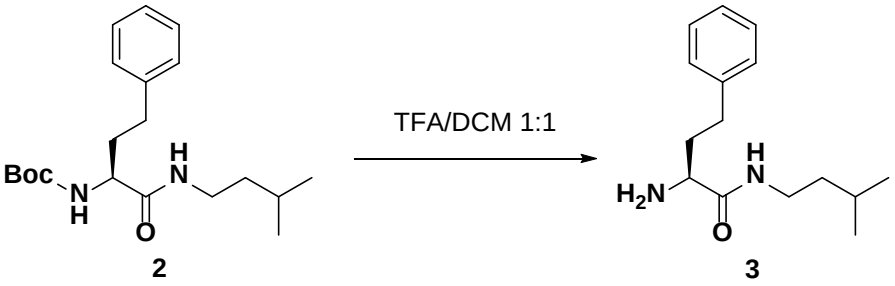
Synthesis

Compound 2:



Experimental: Boc-Homophenylalanine **1** (500 mg, 1.78 mmol) was dissolved in 8 mL DMF. To this solution was added HATU (741 mg, 3.56 mmol) and DIPEA (929 µL, 5.34 mmol) in succession. The resulting mixture was stirred for 30 minutes before addition of isopentylamine (412 µL, 3.56 mmol). The solution quickly turned yellow and the mixture was allowed to stir for 14 h. The mixture was diluted with EtOAc (30 mL), washed with water (2x20 mL), sat. $NaHCO_3$ (2x20 mL), and brine (20 mL). The organic layer was collected, dried over $MgSO_4$, and filtered. The clear yellow solution was concentrated under reduced pressure, and the yellow residue was purified by column chromatography (2:1 Hexanes/EtOAc). The product **2** was isolated as a clear, colorless foam (365 mg, 58% yield). **^{1}H NMR** (300 MHz, Chloroform-*d*) δ 7.30 – 7.12 (m, 6H), 6.78 (s, 1H), 5.59 (d, *J* = 7.9 Hz, 1H), 4.26 – 4.14 (m, 1H), 3.22 (s, 2H), 2.74 – 2.62 (m, 2H), 2.01 – 1.90 (m, 1H), 1.62 (dt, *J* = 13.2, 6.7 Hz, 1H), 1.47 (s, 14H), 1.12 (s, 1H), 0.91 (d, *J* = 6.6 Hz, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 172.18, 156.10, 141.36, 128.70, 128.62, 126.29, 80.10, 77.63, 77.37, 77.12, 54.42, 38.61, 38.01, 34.67, 32.18, 28.61, 26.02, 22.72, 22.67. **HRMS** (ESI) *m/z*: calculated for $C_{20}H_{33}N_2O_3$ $[M+H]^+$ 349.2486, found 349.2481. **R_f**: 0.47 (2:1 Hexanes/EtOAc).

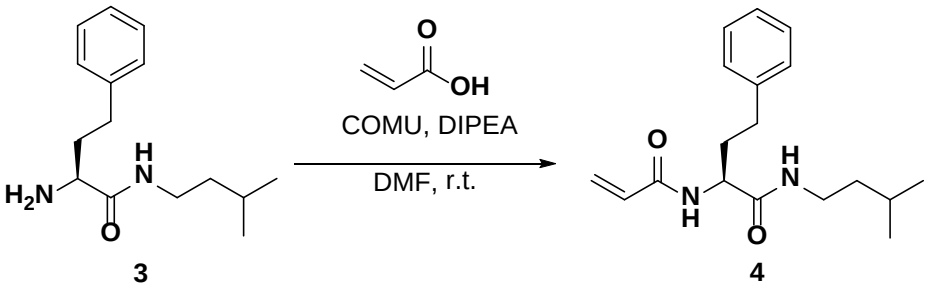
Compound 3:



Experimental: Compound **2** (360 mg, 1.03 mmol) was dissolved in DCM (10 mL) with stirring, and TFA (10 mL) was added to the solution. The mixture was stirred until complete disappearance of the starting material by TLC (2:1 Hexanes/EtOAc, ~20 min). The solvent was removed under reduced pressure, and the residue was dissolved in DCM (20 mL) and washed with saturated $NaHCO_3$ (3x15 mL). The combined aqueous layers were back extracted with DCM (2x20 mL). The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting yellow oil was found to be compound **3** and was used without further purification (247.9 mg, 97%). **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.29 (d, *J* = 7.6 Hz, 1H),

7.24 – 7.13 (m, 4H), 3.37 (dd, *J* = 8.4, 3.9 Hz, 1H), 3.27 (q, *J* = 6.8 Hz, 2H), 2.78 – 2.67 (m, 2H), 2.26 – 2.13 (m, 1H), 1.88 – 1.75 (m, 1H), 1.60 (s, 4H), 1.40 (q, *J* = 7.5 Hz, 2H), 0.92 (d, *J* = 6.6 Hz, 6H).

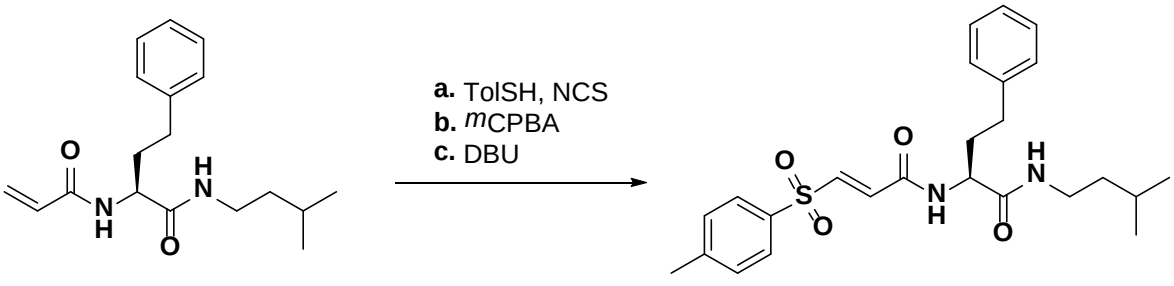
Compound 4:



3 **4**

Experimental: Compound **3** (50 mg, 0.20 mmol) was dissolved in DMF (2.0 mL), and to this the acrylic acid (13.7 µL, 0.20 mmol) and DIPEA (104 µL, 0.60 mmol) were added with stirring. The mixture was cooled to 0 °C and COMU (86 mg, 0.20 mmol) was added. The flask was backfilled with N_2 and allowed to stir for 1 h. At this time the mixture was warmed to room temperature and stirred for another 3 hours. The mixture was diluted with EtOAc (10 mL) and washed with 1 N HCl (10 mL), sat. $NaHCO_3$ (2x10 mL), and brine (10 mL). The organic layer was dried over $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The yellow residue was purified by column chromatography (2:1 to 3:2 to 1:1 Hexanes/EtOAc). The product **4** was isolated as a white powder (34.3 mg, 57%). **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.23 (dd, *J* = 13.5, 6.5 Hz, 1H), 7.14 (dd, *J* = 19.9, 6.8 Hz, 4H), 6.85 (d, *J* = 4.6 Hz, 1H), 6.32 – 6.11 (m, 2H), 5.64 (d, *J* = 9.9 Hz, 1H), 4.63 (q, *J* = 7.3 Hz, 1H), 3.34 – 3.26 (m, 1H), 3.19 (dt, *J* = 13.1, 6.5 Hz, 1H), 2.67 (t, *J* = 8.0 Hz, 2H), 2.16 (dq, *J* = 14.6, 8.1 Hz, 1H), 2.04 (dt, *J* = 13.9, 7.2 Hz, 1H), 1.60 (dt, *J* = 13.2, 6.8 Hz, 1H), 1.39 (q, *J* = 7.2 Hz, 2H), 0.89 (d, *J* = 6.6 Hz, 5H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.73, 165.75, 141.20, 130.78, 128.70, 128.58, 127.11, 126.30, 77.59, 77.27, 76.95, 53.29, 38.48, 38.16, 34.60, 32.11, 26.05, 22.70, 22.61. **HRMS** (ESI) *m/z*: calculated for $C_{18}H_{27}N_2O_2$ $[M+H]^+$ 303.2067, found 303.2070. **R_f** 0.4 (1:1 Hexanes/EtOAc).

WRR139:



4 **WRR139**

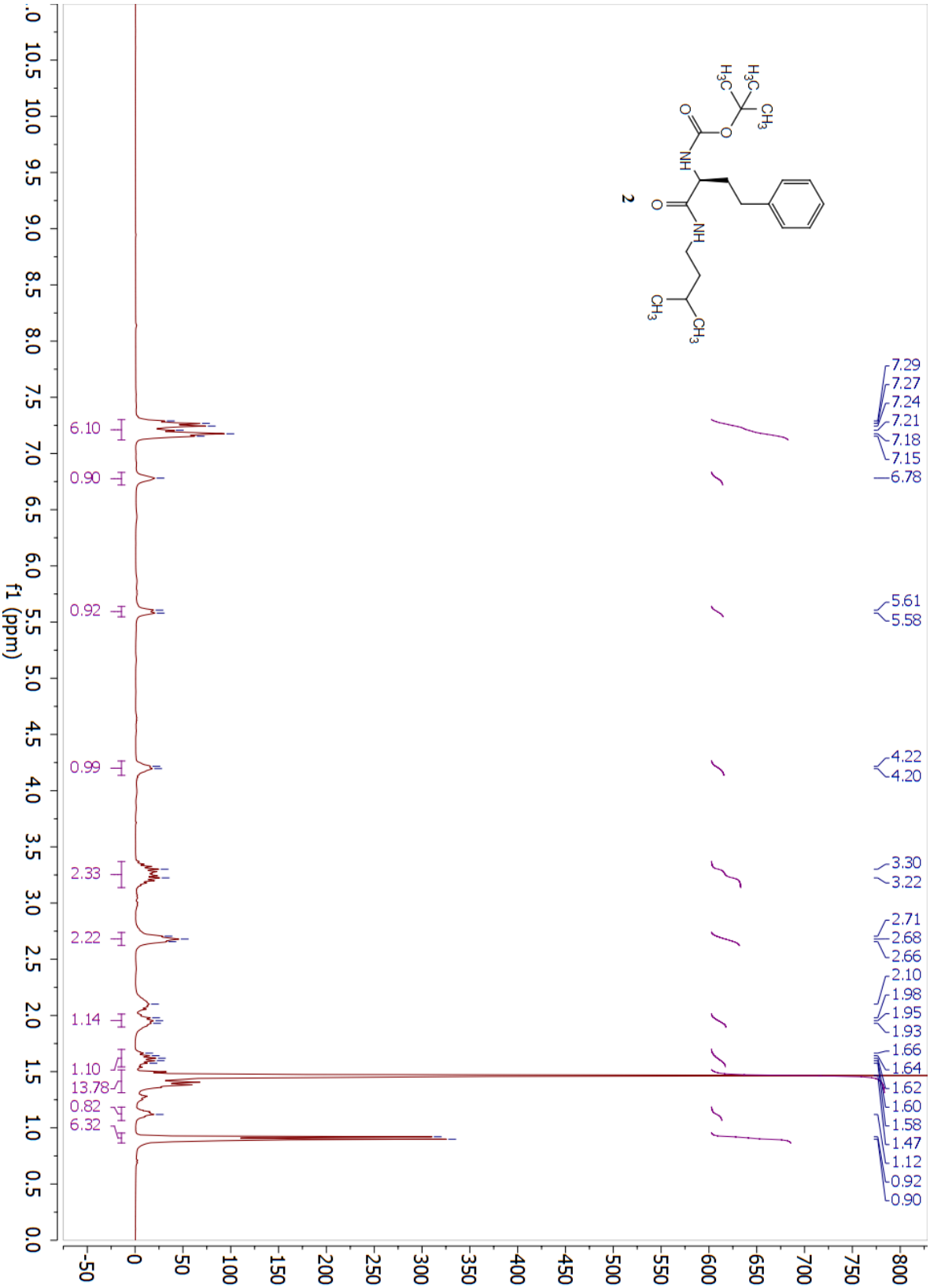
Experimental:

Addition: N-chlorosuccinimide (32.3 mg, 0.242 mmol) was dissolved in DCM (3 mL), and *p*-methylbenzenethiol (28.6 mg, 0.231 mmol) was added slowly with stirring. The solution turned orange and a precipitate formed. The mixture was allowed to stir for 30 minutes. Compound **4** was separately dissolved in DCM (0.25 mL) and added slowly. The reaction mixture was stirred for 14 h, then diluted with DCM (10 mL), washed with water (10 mL), and finally brine (10 mL).

The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was used in the subsequent step without purification.

Oxidation: The crude residue obtained from the addition was dissolved in DCM (4 mL) and the solution was cooled to 0 °C. The *m*CPBA (113 mg, 0.508 mmol) was added slowly, and the reaction was allowed to warm to r.t. and stirred. The reaction was monitored by TLC (the intermediate sulfoxide can be seen during this transformation, very polar), and after 1 h a second equivalent of *m*CPBA (113 mg, 0.508 mmol) was added due to incomplete conversion. The reaction mixture was stirred for 1 h before being quenched with sat. sodium thiosulfate (5 mL). The mixture was diluted with DCM (10 mL), and the organic layer was separated and washed with sat. $NaHCO_3$ (10 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. After thorough drying under high vacuum, the chunky white residue was used directly in the subsequent step without purification.

Elimination: The crude residue from the oxidation was dissolved in DCM (3.2 mL) and cooled to 0 °C. To this solution was added DBU (50 µL, 0.33 mmol) dropwise, and the mixture was allowed to warm to r.t. After stirring for 15 min, the mixture was diluted with DCM (10 mL), washed with 1N HCl (10 mL), water (10 mL), and finally brine (10 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting crude material was purified using a Biotage Isolera Prime with two columns (SiO_2, 10-80% EtOAc in hexanes, then 0-8% MeOH in DCM). A white residue (8.9 mg, 8.4% over three steps) was obtained. **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.77 (dt, *J* = 8.3, 1.9 Hz, 2H), 7.61 – 7.48 (m, 1H), 7.37 – 7.28 (m, 3H), 7.28 – 7.20 (m, 2H), 7.17 (dt, *J* = 9.1, 4.8 Hz, 1H), 7.10 (d, *J* = 7.5 Hz, 2H), 7.02 (dt, *J* = 14.7, 2.0 Hz, 1H), 6.15 (d, *J* = 6.6 Hz, 1H), 4.50 (q, *J* = 7.0 Hz, 1H), 3.27 (ddd, *J* = 40.4, 13.6, 6.7 Hz, 2H), 2.64 (s, 2H), 2.43 (s, 3H), 2.15 (dd, *J* = 14.2, 7.1 Hz, 1H), 2.00 (dq, *J* = 14.7, 7.4 Hz, 1H), 1.57 (dq, *J* = 13.5, 6.7 Hz, 1H), 1.43 – 1.32 (m, 2H), 0.88 (dd, *J* = 6.5, 1.8 Hz, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.16, 162.51, 145.54, 141.05, 140.65, 136.03, 133.67, 133.18, 130.37, 130.06, 128.83, 128.69, 128.57, 128.55, 126.52, 77.54, 77.28, 77.03, 53.61, 38.40, 38.34, 34.43, 32.02, 26.03, 22.68, 22.61, 21.96. **HRMS** (ESI) *m/z*: calculated for $C_{25}H_{33}N_2O_4S$ $[M+H]^+$ 457.2156, found 457.2146. **R_f** 0.32 (3% MeOH in DCM).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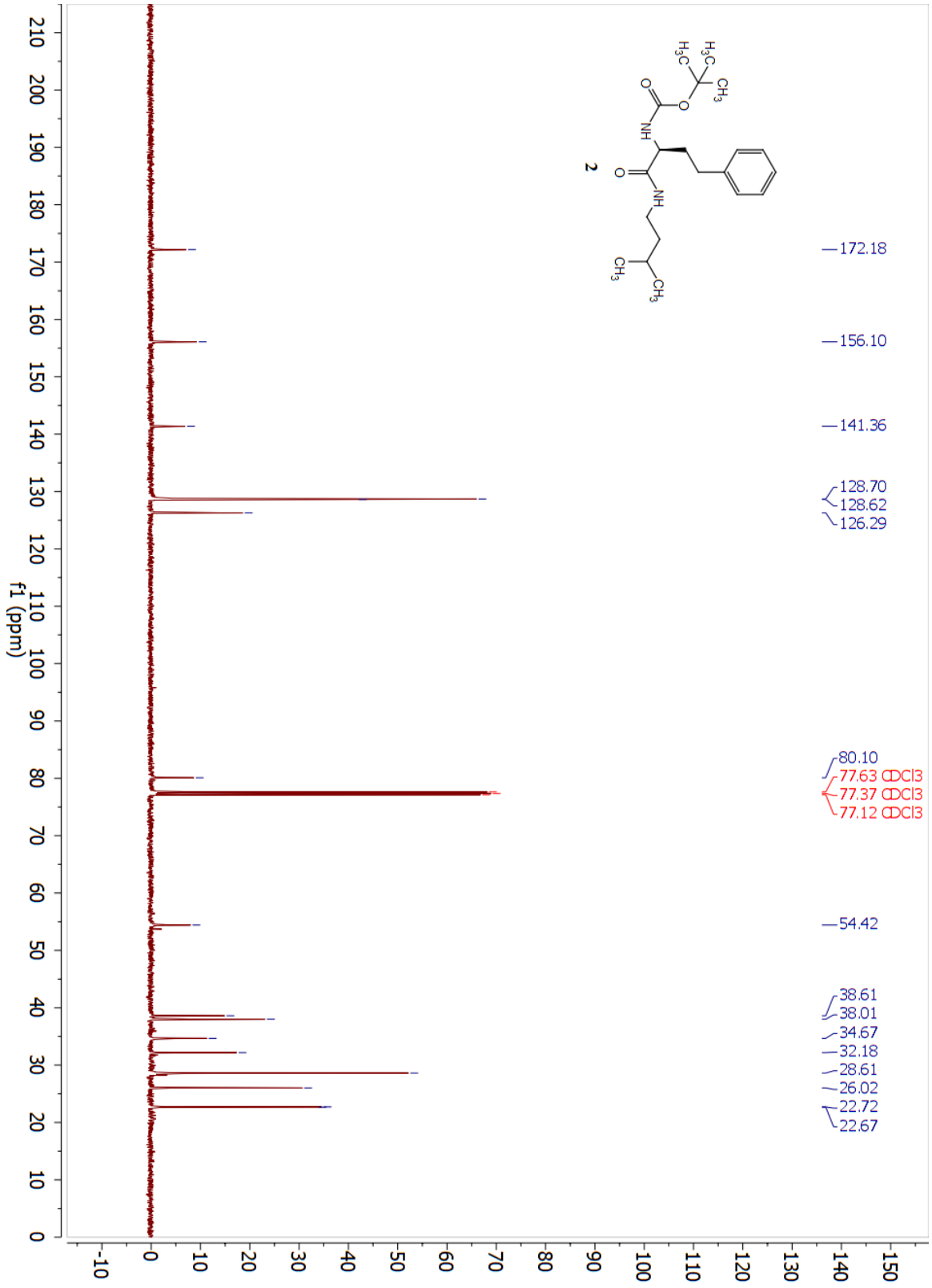
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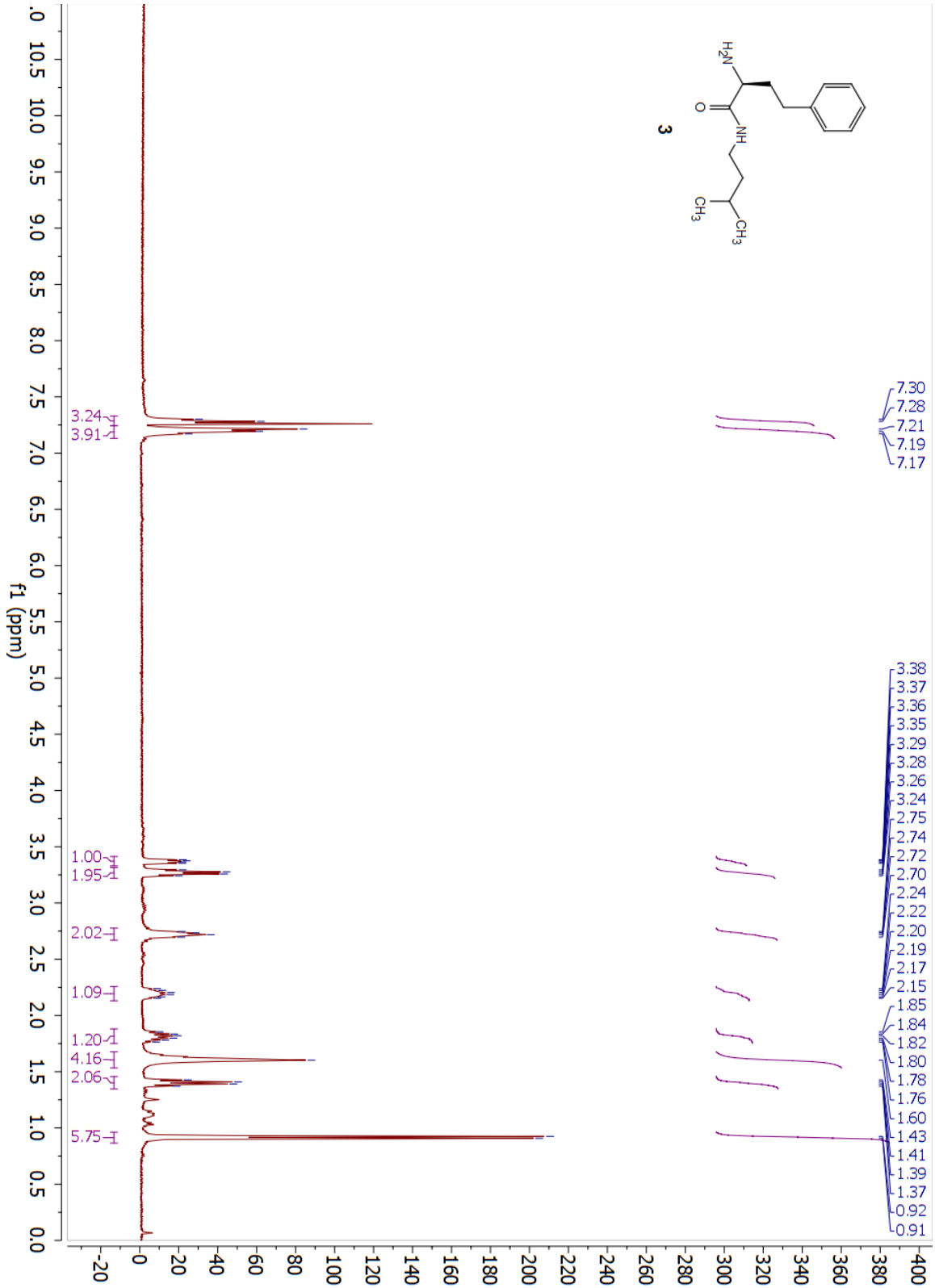
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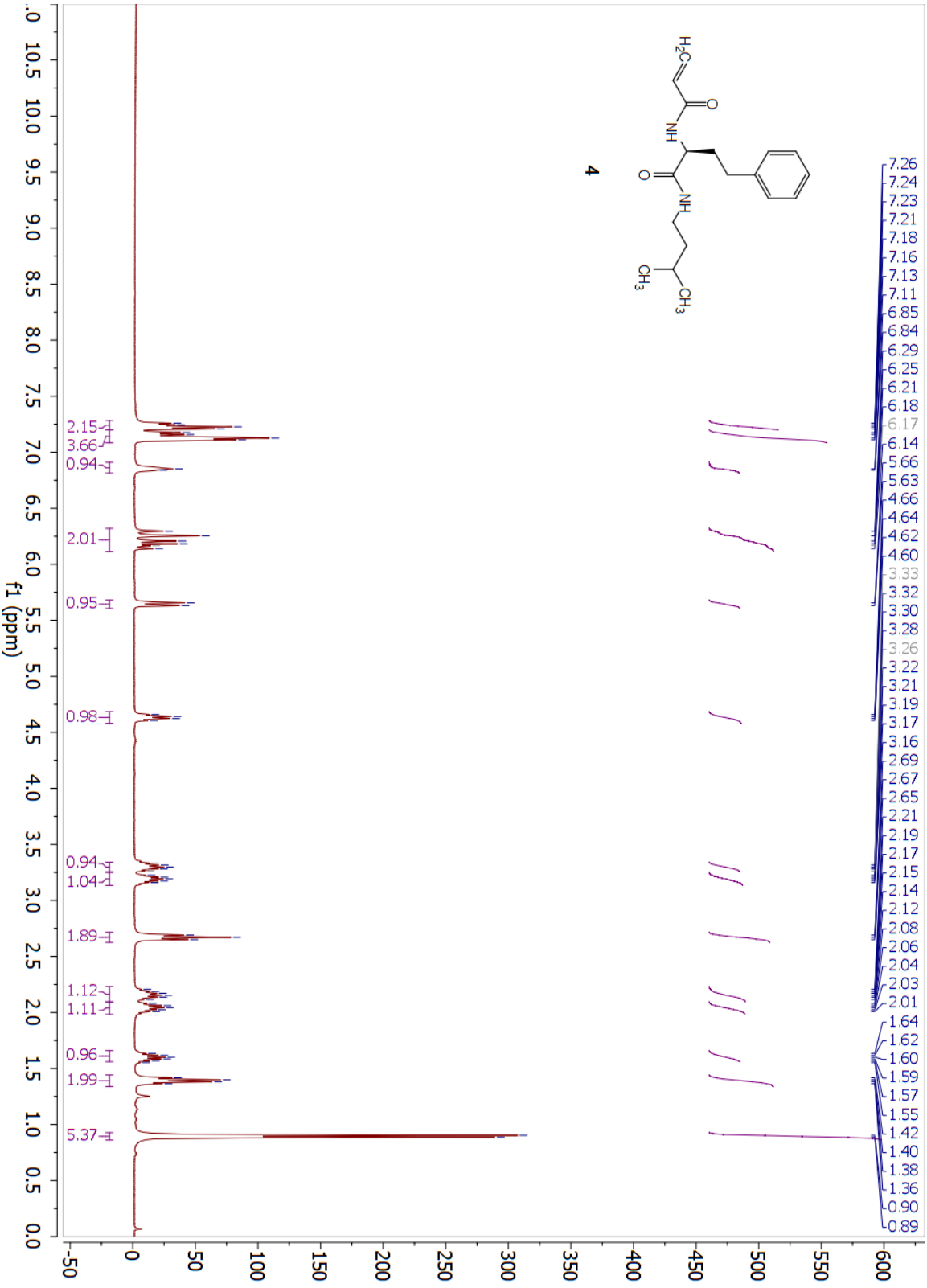
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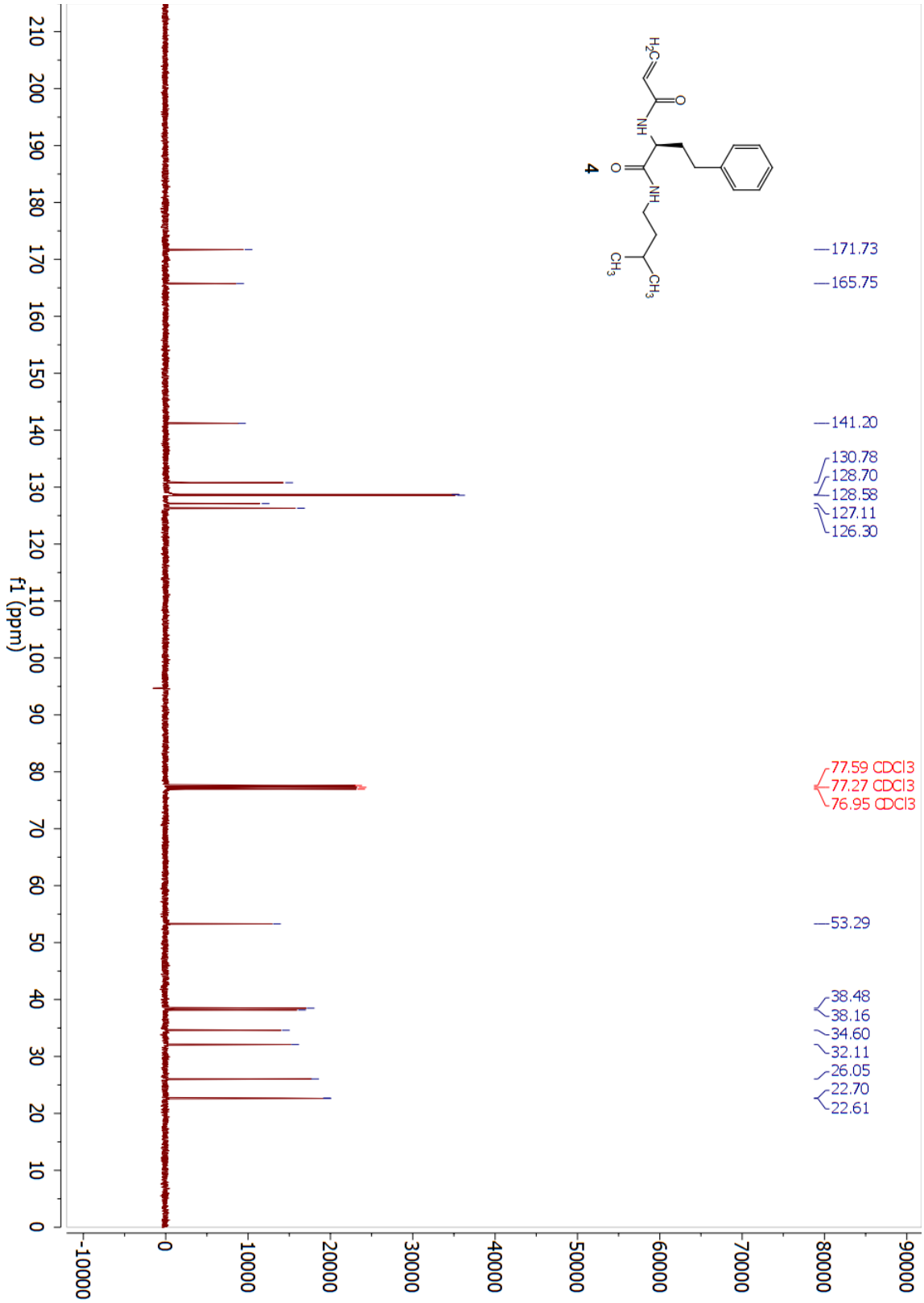
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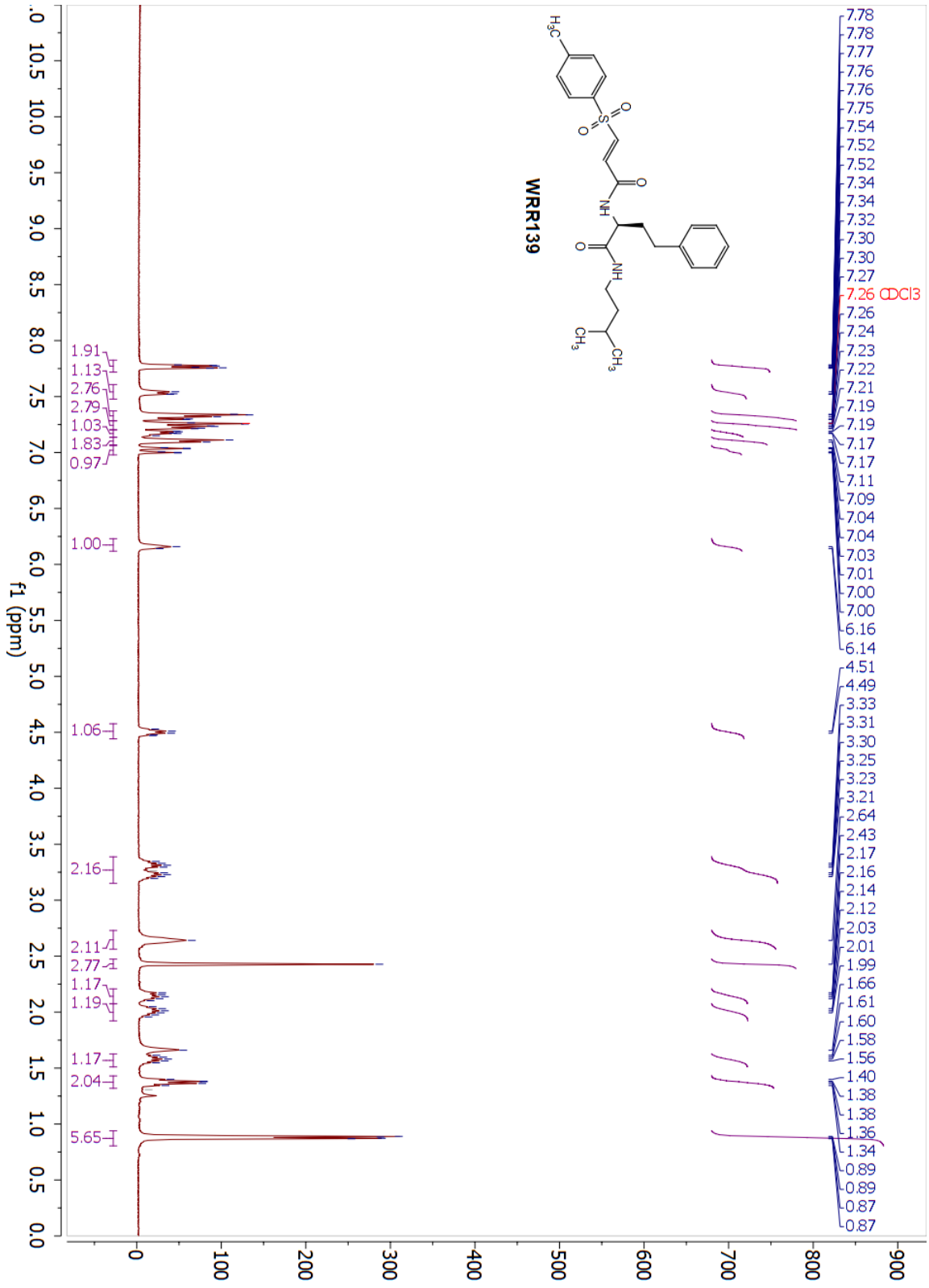
f1 (ppm)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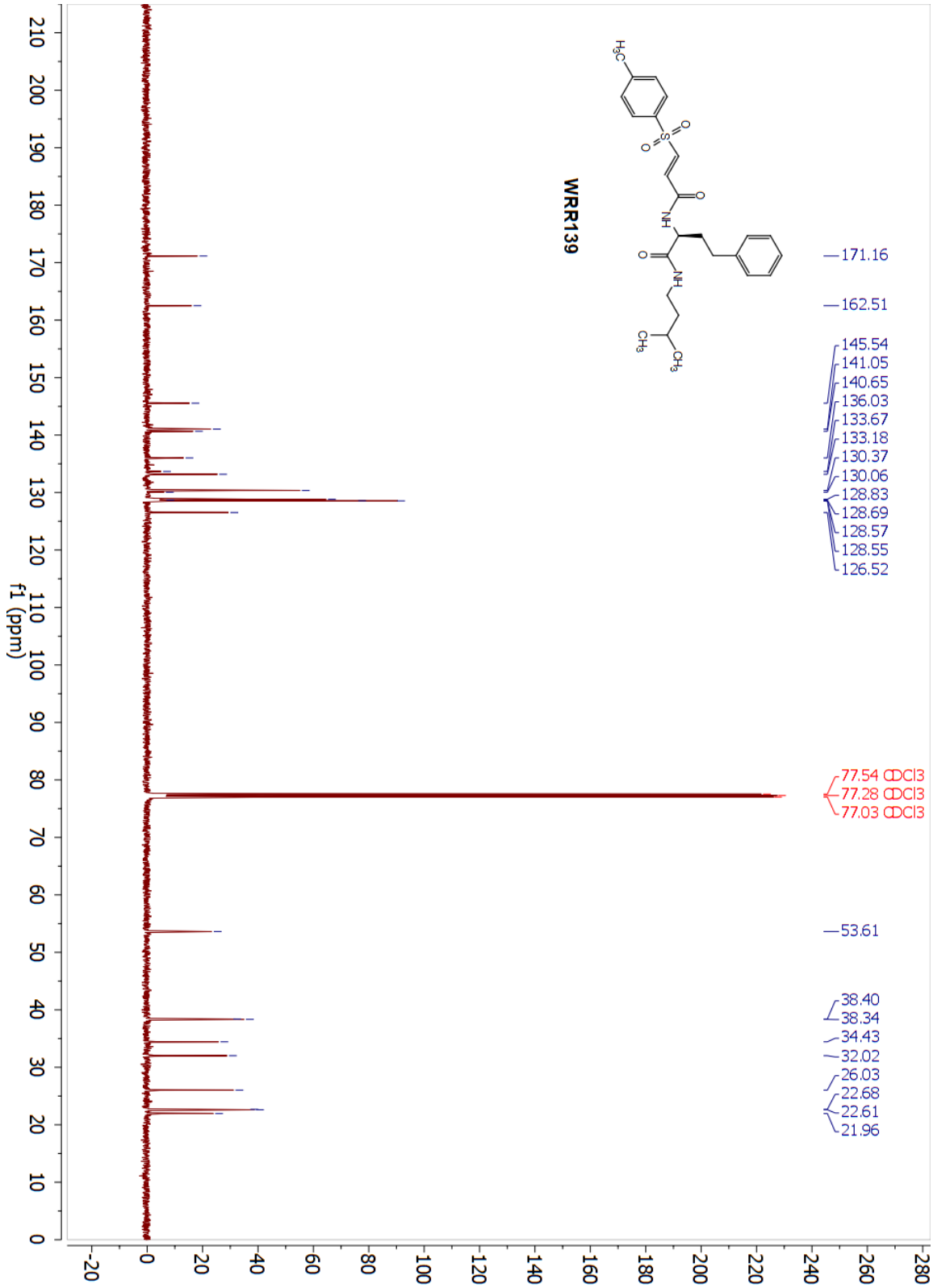
0.87
0.87


WRR139
171.16
162.51
145.54
141.05
140.65
136.03
133.67
133.18
130.37
130.06
128.83
128.69
128.57
128.55
126.52
77.54 CDCl3
77.28 CDCl3
77.03 CDCl3
53.61
38.40
38.34
34.43
32.02
26.03
22.68
22.61
21.96
f1 (ppm)