Supporting Information

When Tight is too Tight: Dasatinib and its Lower Affinity Analogue for Profiling Kinase Inhibitors in a Three-Hybrid Split-luciferase System

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Table S1. Kinase constructs

Kinase	**Uniprot Ref.Seq.**
ABL	P00519
PTK6	Q13882
Src	P12931
EPHB3	P54753
RIPK2	O43353
EPHA2	P29317

Table S2. Luciferase constructs

Construct	Reference
Nfluc	Porter, J. *et al* JACS, **130**, 6488 (2008).
Cfluc	Porter, J. *et al* JACS, **130**, 6488 (2008).
Fos-Nfluc	Porter, J. *et al* JACS, **130**, 6488 (2008).
Cfluc-Jun	Porter, J. *et al* JACS, **130,** 6488 (2008).

Table S3. Normalized % Inhibition Values for 72 Inhibitors Tested Against ABL.

Inhibitor	Row	Column	% Inhibition
SB 218078	A	1	21%
PKC-412	A	2	9%
Ro 31-8220 mesylate	A	3	15%
GF 109203X	A	4	8%
Arcyriaflavin A	A	5	0%
PD 407824	A	6	3%
CGP 53353	A	7	9%
SB 216763	A	8	6%
SB 415286	A	9	6%
Sunitinib	B	1	33%
GW 5074	B	2	9%
PHA 665752	B	3	27%
GW 843682X	B	4	14%
Ki 8751	B	5	92%
IKK 16	B	6	53%
ZM 447439	B	7	12%
Iressa	B	8	5%
ZM 306416 hydrochloride	B	9	35%
GW 583340 dihydrochloride	C	1	10%
ZM 323881 hydrochloride	C	2	0%
BIBX 1382 dihydrochloride	C	3	4%
ZM 39923 hydrochloride	C	4	0%
ZM 449829	C	5	0%
ZM 336372	C	6	40%
SD 208	C	7	11%
LY 364947	C	8	13%
LY 294002 hydrochloride	C	9	21%
NU 7026	D	1	4%
PI 828	D	2	6%
Compound 401	D	3	0%
Purvalanol A	D	4	28%
Purvalanol B	D	5	22%
Aminopurvalanol A	D	6	45%
PP 1	D	7	39%
PP 2	D	8	41%
1-Naphthyl PP1	D	9	23%

Inhibitor	Row	Column	% Inhibition
CGP 57380	E	1	17%
Roscovitine	E	2	6%
Olomoucine	E	3	0%
API-2	E	4	5%
TPCA-1	E	5	17%
SC 514	E	6	0%
TBB	E	7	6%
U0126	E	8	23%
AG 213	E	9	0%
AG 490	F	1	0%
LFM-A13	F	2	0%
SL 327	F	3	0%
IMD 0354	F	4	1%
Flavopiridol	F	5	3%
Rottlerin	F	6	36%
Arctigenin	F	7	4%
10-DEBC hydrochloride	F	8	2%
ER 27319 maleate	F	9	10%
SP 600125	G	1	0%
H 89 dihydrochloride	G	2	2%
HA 1100 hydrochloride	G	3	0%
Fasudil hydrochloride	G	4	0%
ML 9 hydrochloride	G	5	0%
Y-27632 dihydrochloride	G	6	1%
EO 1428	G	7	12%
Ro 08-2750	G	8	6%
PD 198306	G	9	1%
SB 202190	H	1	0%
SB 431542	H	2	9%
D 4476	H	3	10%
SB 203580 hydrochloride	H	4	0%
SB 239063	H	5	2%
CGK 733	H	6	6%
PQ 401	H	7	0%
(-)-Terreic acid	H	8	0%
1,2,3,4,5,6-Hexabromocyclohexane	H	9	0%

General experimental information

Reagents and material. 2-amino-*N*-(2-chloro-6-methylphenyl)thiazole-5-carboxamide and 1-(2-NBoc-aminoethyl)-piperazine were purchased from *AK Scientific*. 2-((6-chloro-2-methylpyrimidin-4-yl)amino)-*N*-(2-chloro-6-methylphenyl)thiazole-5-carboxamide was obtained from Combi Blocks. Staurosporine was either purchased from LC Laboratories or produced from *Lentza albida.* Enzymes and oligonucleotide primers for cloning were obtained from NEB and IDT respectively. $Mg(OAc)_2$ was obtained from EM Sciences and Tris HCl was obtained from Research Products International Corp. All translation materials were either from Promega or Luceome Biotechnologies. All other reagents were purchased from Sigma-Aldrich. H-NMR spectra were obtained from Brucker 400 and Brucker 500 at the University of Arizona. Chemical shifts are reported in δ ppm relative to tetramethylsilane. Coupling constants (*J*) are reported in Hertz (Hz).

Clone Construction and mRNA Synthesis

Fos-Nfluc and Cfluc-kinase fusions were cloned as previously described. In short, PCR products of each kinase domain were prepared with appropriate primers and cloned via restriction endonuclease digestion and ligation into a pRSF-Duet vector containing Cfluc and a multiple cloning site. The resulting Cfluc-kinase constructs contained a 13-residue $(GGS)_n$ linker between the Cfluc and kinase domains. Upon sequence confirmation via Sanger DNA sequencing, linearized fragments of each Cfluc-kinase construct were synthesized with primers encoding a T7 RNA polymerase site, a mammalian Kozak sequence, and a hairpin loop. This linear DNA was subsequently used as a template for *in vitro* mRNA synthesis using Ribomax Large Scale RNA Production System-T7 kits

from Promega. mRNA was purified using Illustra ProbeQuant G-50 micro columns (GE Hearthcare).

Protein Translation and Small Molecule Inhibition Assay:

The *in vitro* transcribed mRNAs encoding Fos-Nfluc and a Cfluc-kinase were transcribed into protein using rabbit reticulocyte lysate from either Luceome Biotechnologies or Promega (Flexi® Rabbit Reticulocyte Lysate System) according to the manufacturer's protocol. Per 25 μL reaction, 0.4 and 1 pmol of Cfluc-kinase and Fos-Nfluc mRNA, respectively, were added to each translation mix. The total volume of translated lysate was scaled up depending on the number of assays. Each translation mix was incubated at 30 ºC for 1.5 h then either used immediately or stored at -80 ºC overnight.

In order to measure inhibition, lysate mixtures were first treated with 1 μL of 7.5 μM chemical inducer of dimerization (CID) per 24 μL of translated lysate, to a final concentration of 300 nM CID, to induce ternary complex formation and luciferase reassembly. In order to quantify background levels of protein association, water was used in place of the CID. For inhibition analysis, 1 μL of 250 μM inhibitor dissolved in DMSO was added to 24 μL aliquots of the lysate plus CID mixture, for a final inhibitor concentration of 10 μM. DMSO, absent an inhibitor, was added used as a control to determine the maximal, uninhibited luminescence. Reactions were incubated at room temperature for one hour prior to measuring luminescence. Luminescence was measured by first mixing 20 μL of lysate aliquots with 80 μL luciferin assay reagent, incubating for 1 minute, and then reading each sample with a 10 s integration time on a Turner Biosystems 20/20n luminometer.

Small-Molecule Inhibitor Profiling and Percent Inhibition Calculation

The plate-based profiling for ABL followed the method described above with a few modifications. After addition of CID to translated lysate containing Fos-Nfluc and Cfluc-ABL, 24 μL aliquots of lysate were added to each well of a 96-well Lumitrac 200 plate (Greiner Bio-one) containing 1 μL of 250 μM inhibitor stock solution in DMSO. The plate was covered and incubated at room temp for 1 h to allow equilibration. A Centros XS LB 960 plate reader (Berthold Technologies) was used to auto-inject 80 μL of luciferin assay reagent into each well and measure luminescence using a 1 s integration time.

For each kinase assay, luminescence data was normalized by first subtracting the background signal for a negative control (in the absence of CID) from both a positive control (with CID but no inhibitor) as well as samples containing both CID and inhibitor. The background subtracted positive control was then scaled to a value of 1, with the inhibitor samples adjusted according. Calculations for the percent inhibition values shown for the ABL screen were similarly normalized, except each normalized value was subtracted from 100%. Additionally, percent inhibition values measured for a Fos-Nfluc, Cfluc-Jun dimer were subtracted from the corresponding inhibition values for ABL in order to account for off-target inhibition of luciferase activity. Percent inhibition values calculated to be < 0 were adjusted to 0%.

Time dependent inhibition study

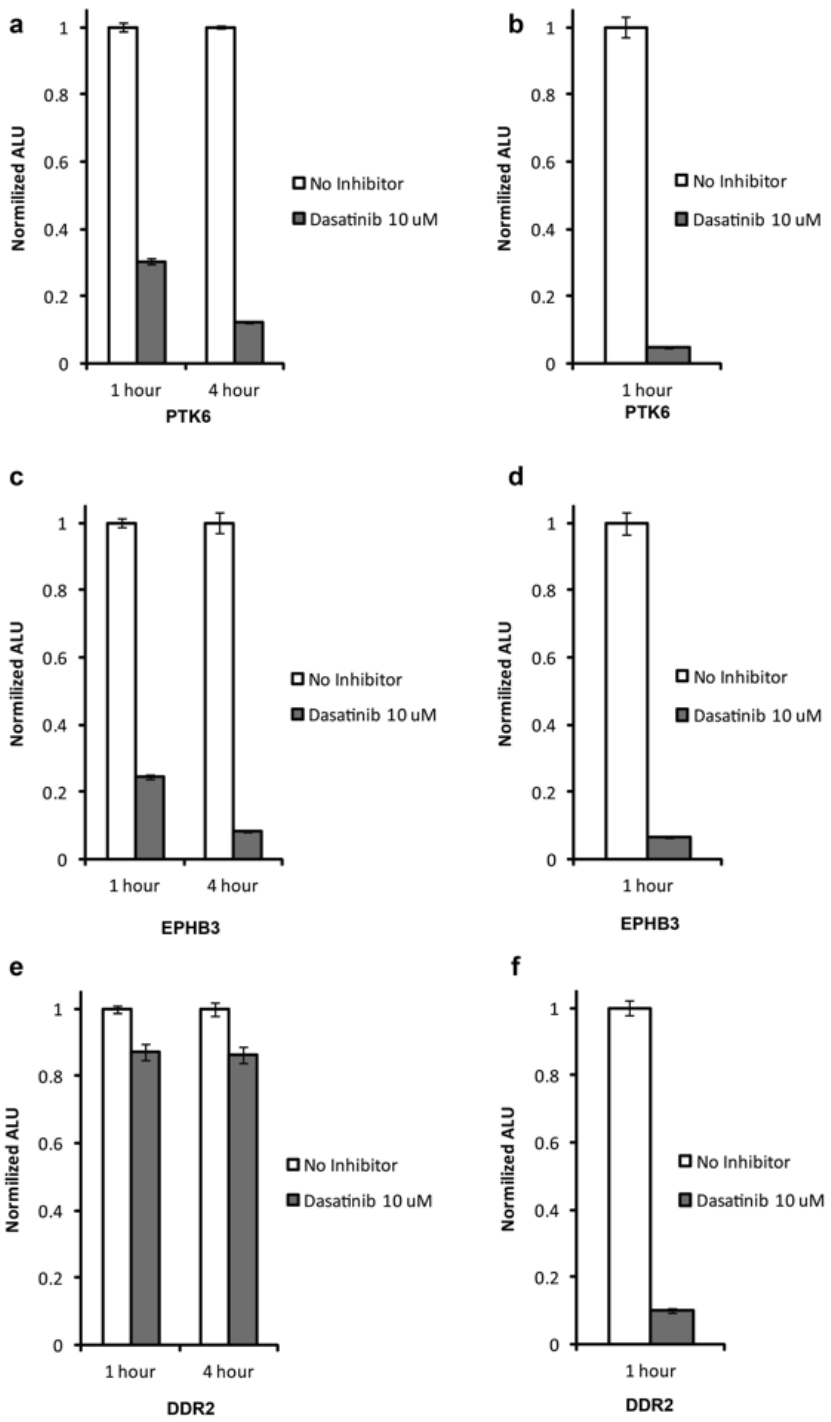
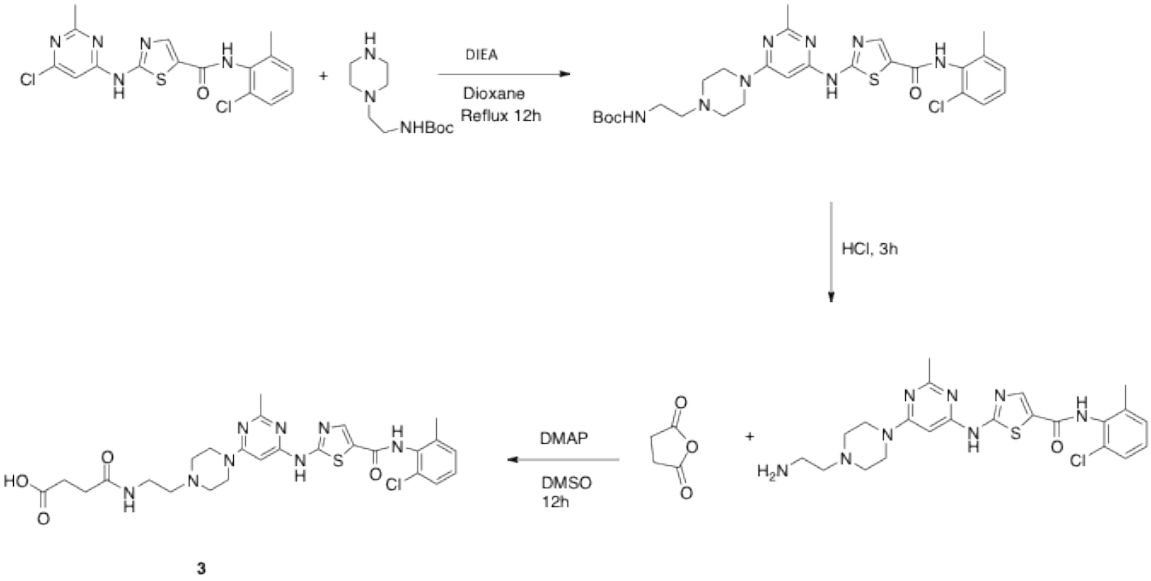


Figure S1 Inhibition of ternary complex for PTK6, EPHB3, and DDR2 using Jun-Dasatinib CID and carboxy-Dasatinib as a competitive ligand. Inhibition assays were preformed for A)PTK6, C)EPHB3, and E)DDR2 where Jun-carboxy-Dasatinib was incubated for 30 mins in the lysate mixture prior to the addition of the carboxy-Dasatinib inhibitor or DMSO (no inhibitor control). Then the inhibitor-lysate mixture was incubated for 1 hour and 4 hours to observe the time dependence of signal inhibition. Inhibition assays for B) PTK6, D) EPHB3, and F)DDR2 were performed by adding Jun-carboxy-Dasatinib and carboxy-Dasatinib inhibitor or DMSO together to the lysate mixure and incubated for 1 hour.

Synthesis

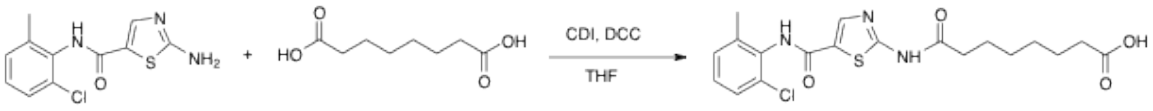
Synthesis of compound **3**[3]



2-((6-chloro-2methylpyrimidin-4-yl)amino)-*N*-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (30 mg, 0.076 mmol), 1-(2-NBoc-aminoethyl)-piperazine (51 mg 0.22 mmol) and diisopropylethylamine (26 μL, 0.15 mmol) in 1,4-dioxane (8 mL) was heated to reflux for 12 h. The reaction mixture was concentrated under vacuum. The crude was purified through column chromatography on silica gel to yield a brown/yellow solid. Subsequently, the solid was dissolved in a mixture of CH_2Cl_2, MeOH, (1:1) and treated with HCl (12 mmol) and stirred at rt for 30 min. The solution was treated with ether and MeOH to obtain the intermediate deprotected compound 2-((6-(4-(2-aminoethyl)piperazin-1-yl)-2-methylpyrimidin-4-yl)amino)-*N*-(2-chloro-6-methylphenyl)thiazole-5-carboxamide. The deprotected compound (18.3 mg 0.038 mmol) without further purification, succinic anhydride (5.66 mg 0.055 mmol), and

DMAP (2.30 mg 0.018 mmol) in DMSO (1 mL) was stirred at rt for 24 h. The final product **3** was isolated as a white solid and purified from the reaction mixture through HPLC (10 mg, 22% yield); ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.64 (s, 1H), 9.91 (s, 1H), 9.85 (s, 1H), 8.24 (s, 1H), 8.19 (t, *J* = 5.7 Hz, 1H), 7.40 (dd, *J* = 7.7 Hz, 1 Hz, 1H), 7.30-7.24 (m, 2H), 6.16 (s, 1H), 4.40-4.30 (m, 2H), 3.66-3.57 (m, 2H), 3.48-3.42 (m, 2H), 3.27-3.22 (m, 2H), 3.20 (t, *J* = 6 Hz, 2H), 3.15-3.06 (m, 2H), 2.50-2.46 (m, 2H), 2.45 (s, 3H), 2.36 (t, *J* = 6.9 Hz, 2H), 2.23 (s, 3H); ^{13}C NMR (500 MHz, DMSO) δ 173.83, 172.00, 165.43, 162.39, 161.92 159.86, 157.23, 140.76, 138.80, 133.46, 132.41, 129.05, 128.23, 127.03, 125.98, 83.45, 54.94, 50.65, 40.75, 33.55, 29.98, 28.96, 25.55, 18.31; MS-MALDI (M+H calculated for $C_{26}H_{31}ClN_8O_4S$): 587.1950 m/z; found: 587.1952 m/z.

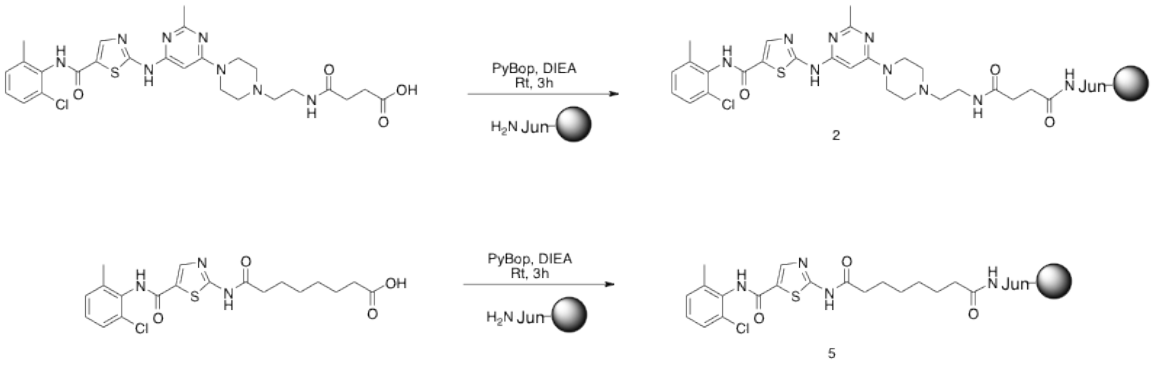
Synthesis of 8-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-8-oxooctanoic acid .



A mixture of CDI (30 mg 0.18 mmol) and DCC (61 mg 0.3 mmol) in THF (2 mL) was kept under stirring at rt for 1 h. 2-amino-*N*-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (100 mg 0.37 mmol) and suberic acid (65 mg 0.37 mmol) in THF (400 μL), was added to the mixture and stirred for 20 h. The resulting suspension was filtered and the filtrate was concentrated under vacuum. The remaining solid was treated with KOH (1 M) and kept under stirring for 30 minutes at rt and filtered. The filtrate was treated with HCl (6 M) resulting in a white suspension, which was stirred for 30 minutes and the solid filtered. The final product was isolated as a white solid and purified through HPLC

(11.4 mg 7% yield); ^{1}H NMR (400 MHz, DMSO) δ 12.40 (s, 1H), 10.03 (s, 1H), 8.28 (s, 1H), 7.41-7.38 (m, 1H), 7.30-7.23 (m, 2H), 2.46 (t, *J* = 7.4 Hz, 2H), 2.23 (s, 3H), 2.19 (t, *J* = 7.4 Hz, 2H), 1.64-1.55 (m 2H), 1.53-1.44 (m 2H), 1.31-1.25 (m 4H); ^{13}C NMR (101 MHz, DMSO) δ 174.51 (s), 171.91 (s), 161.05 (s), 159.53 (s), 140.63 (s), 138.78 (s), 133.33 (s), 132.39 (s), 129.09 (s), 128.33 (s), 127.06 (s), 126.52 (s), 34.87 (s), 33.63 (s), 28.25 (s), 24.45 (s), 24.35 (s), 18.29 (s); MS-MALDI (M+Na calculated for $C_{19}H_{22}ClN_3O_4S$): 446.0912 m/z; found: 446.0908 m/z.

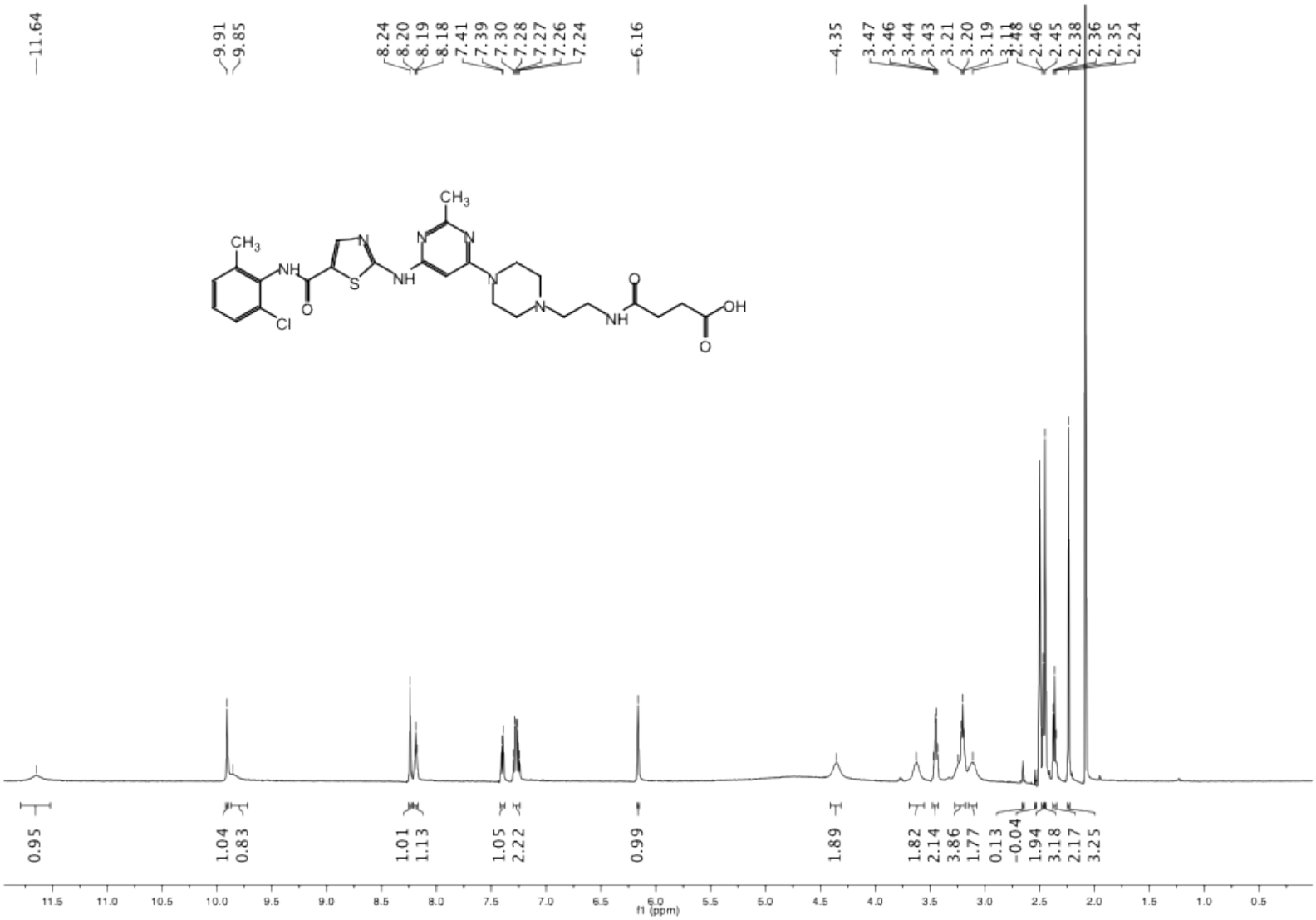
Synthesis of the Jun-conjugated small molecules **2**, **5**:



Jun was synthesized through solid phase peptide synthesis using the Fmoc protection strategy as previously reported. Rink Amide AM resin (0.31 mmol/g) was used as the solid matrix support with PyBop (3 equivalents) and DIEA (6 equivalents) as the coupling agents. The Jun N-terminus was coupled to a linker consisting of two glycines and one β-alanine and the latter amino acid coupled to either carboxy-Dasatinib **3** or the Dasatinib analog. The peptides were cleaved with a solution of TFA (94%), EDT (2.5%), water (2.5%) and TIPS (1%) for 2 h and precipitated with chilled ether following centrifugation, and trituration with additional chilled ether. HPLC purification was

carried out with a 30% to 60% acetonitrile gradient in water with 0.1% TFA. MS/MALDI (calculated for $C_{217}H_{367}N_{70}O_{62}S_2Cl$; **2**): 5048.24 m/z; found 5047.5; MS/MALDI (calculated for $C_{210}H_{358}N_{65}O_{62}S_2Cl$; **5**): 4885.06 m/z; found 4890.89.

Synthesis of **1** has been previously reported[1].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3.47
3.46
3.44
3.43
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3.19
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2.46
2.45
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2.24
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CH3
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NH
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OH
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O
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Cl
S
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N
N
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1.01
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1.05
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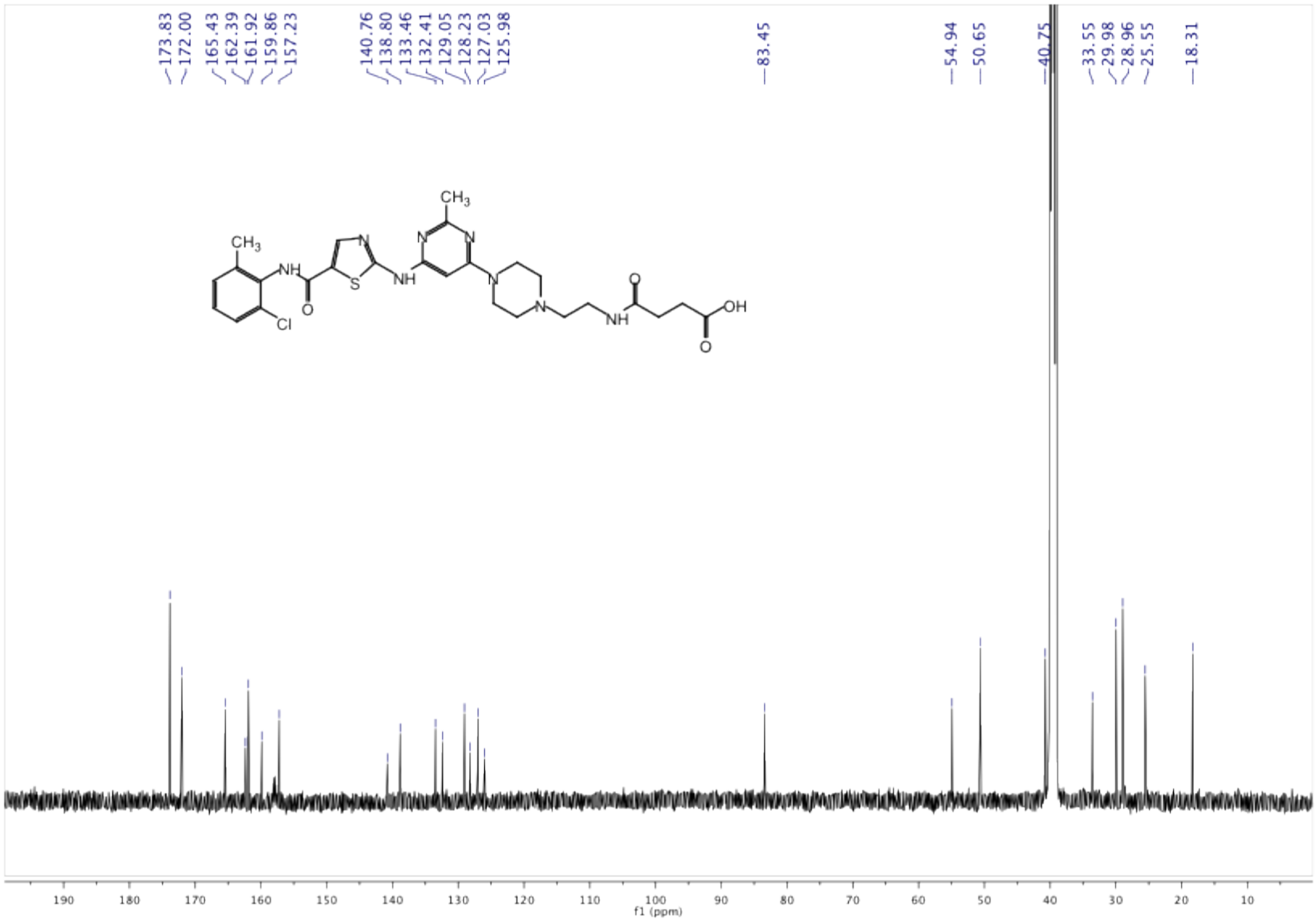
f1 (ppm)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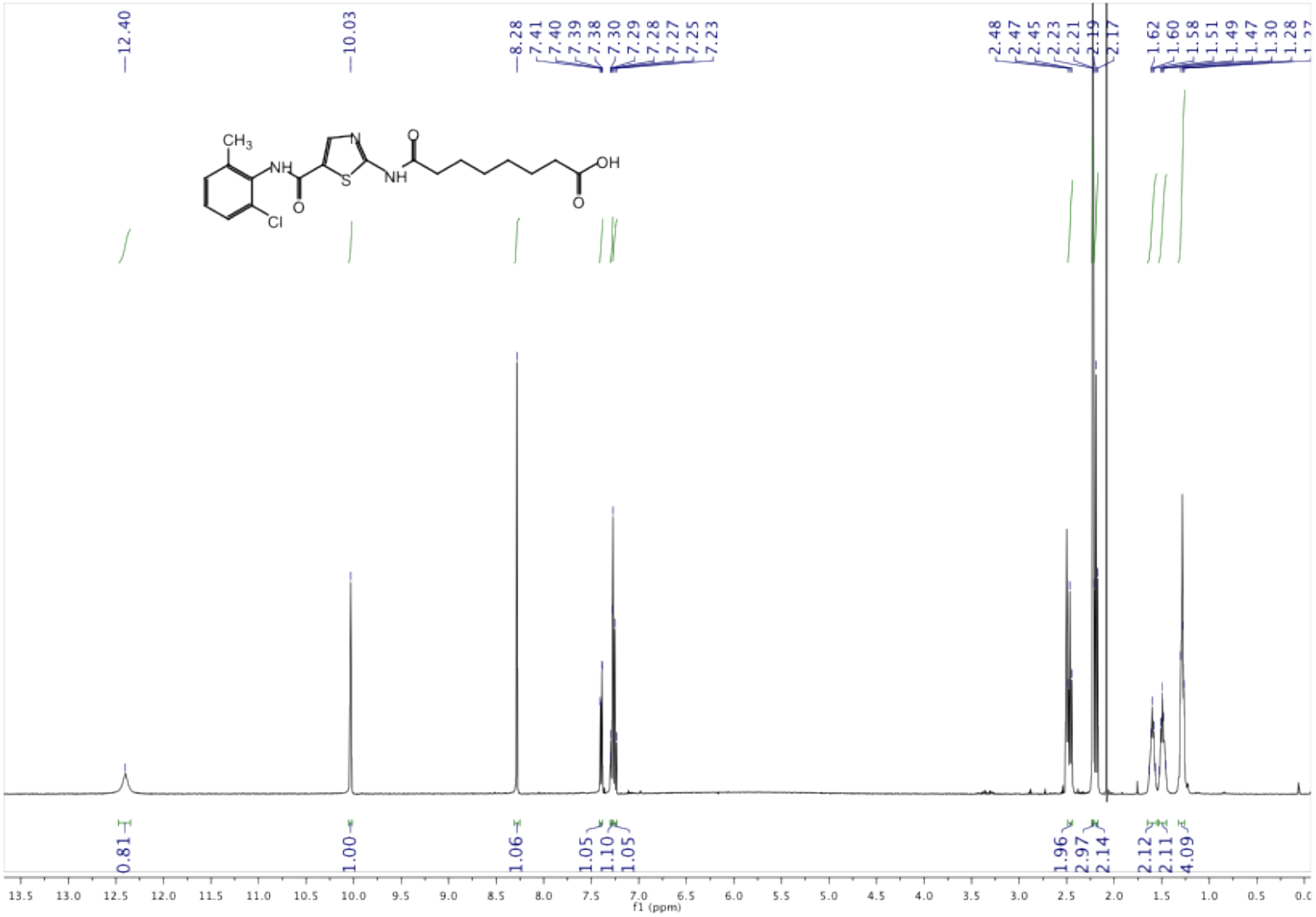
f1 (ppm)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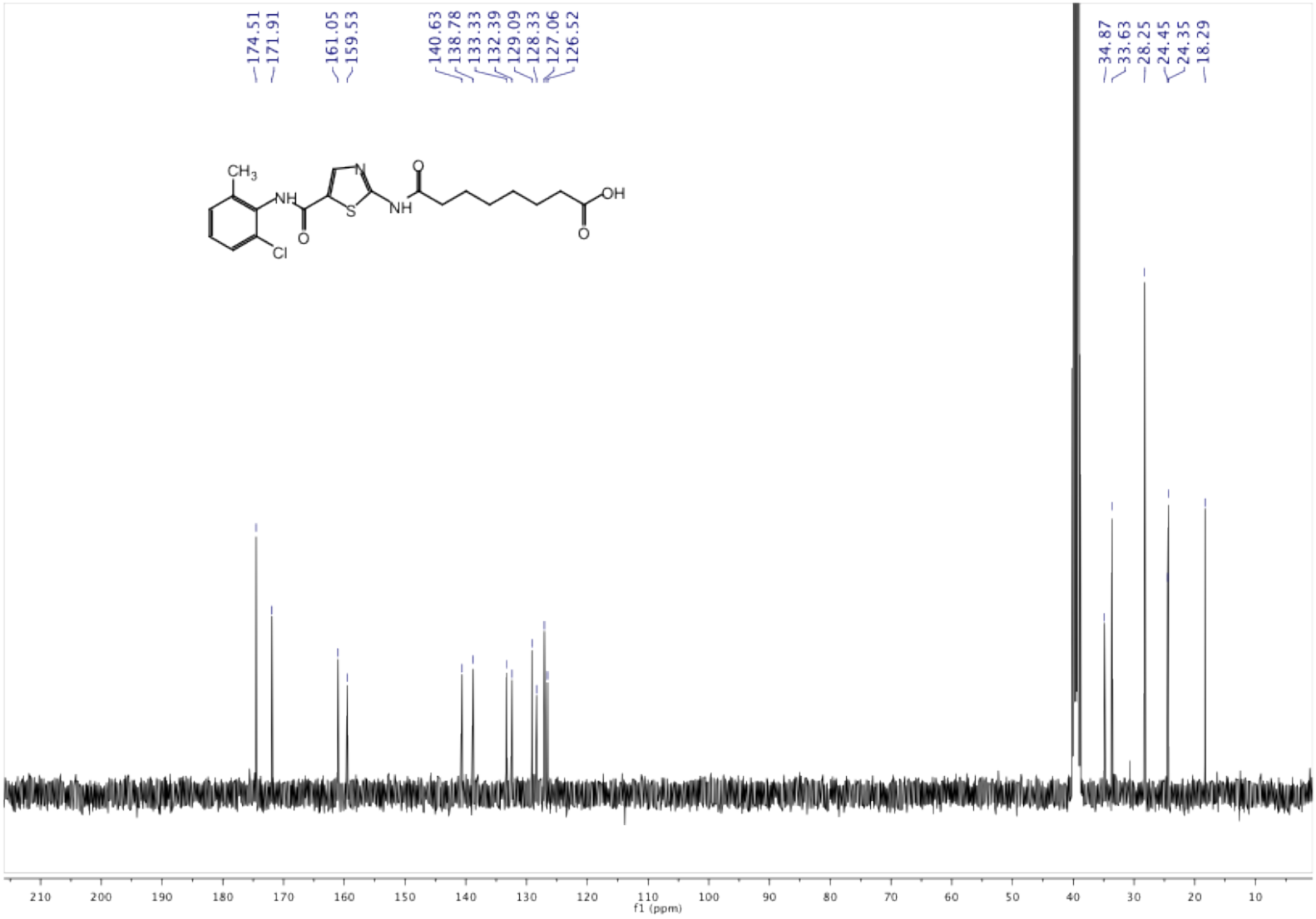
f1 (ppm)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f1 (ppm)

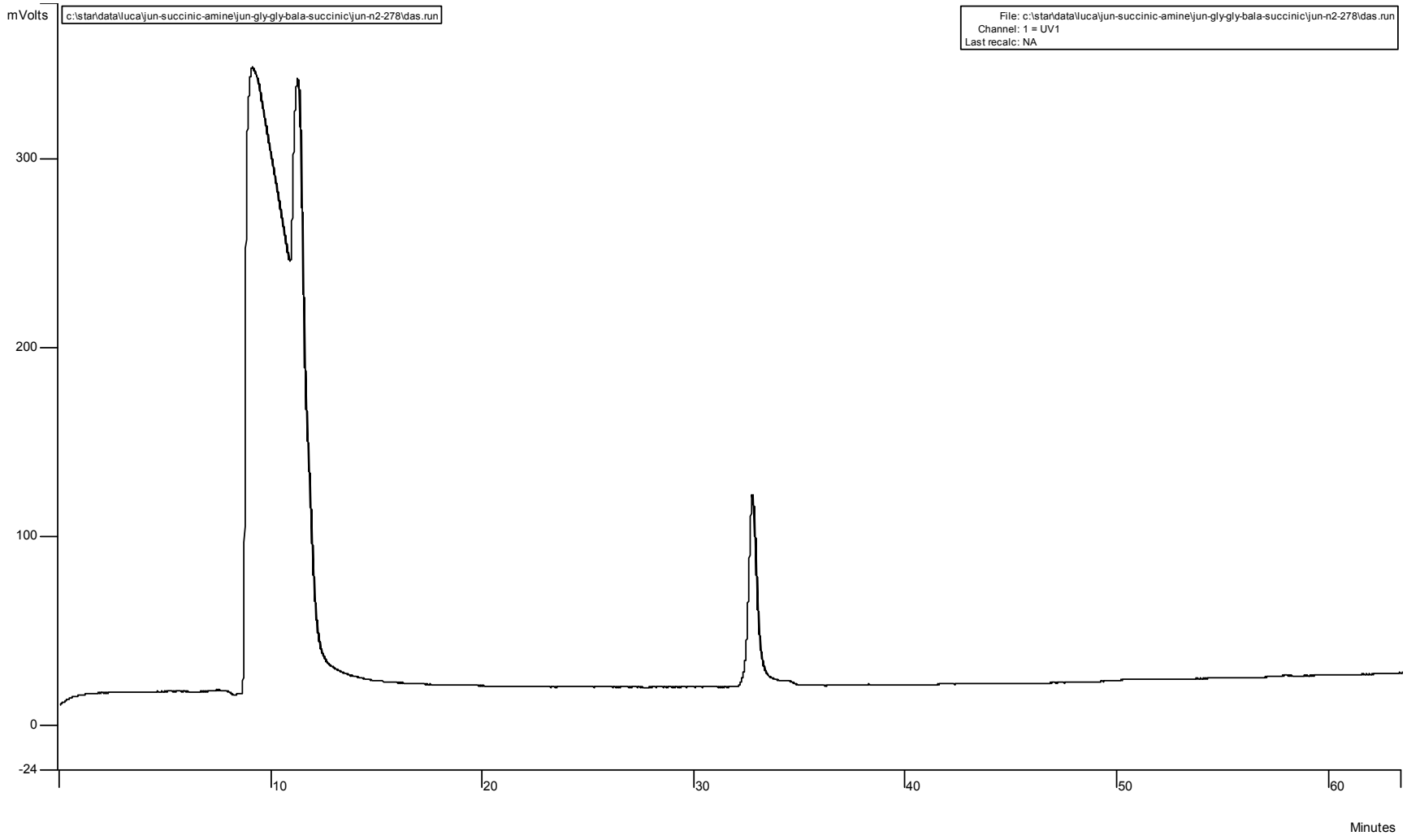

Figure S2 HPLC trace of carboxy-Dasatinib.

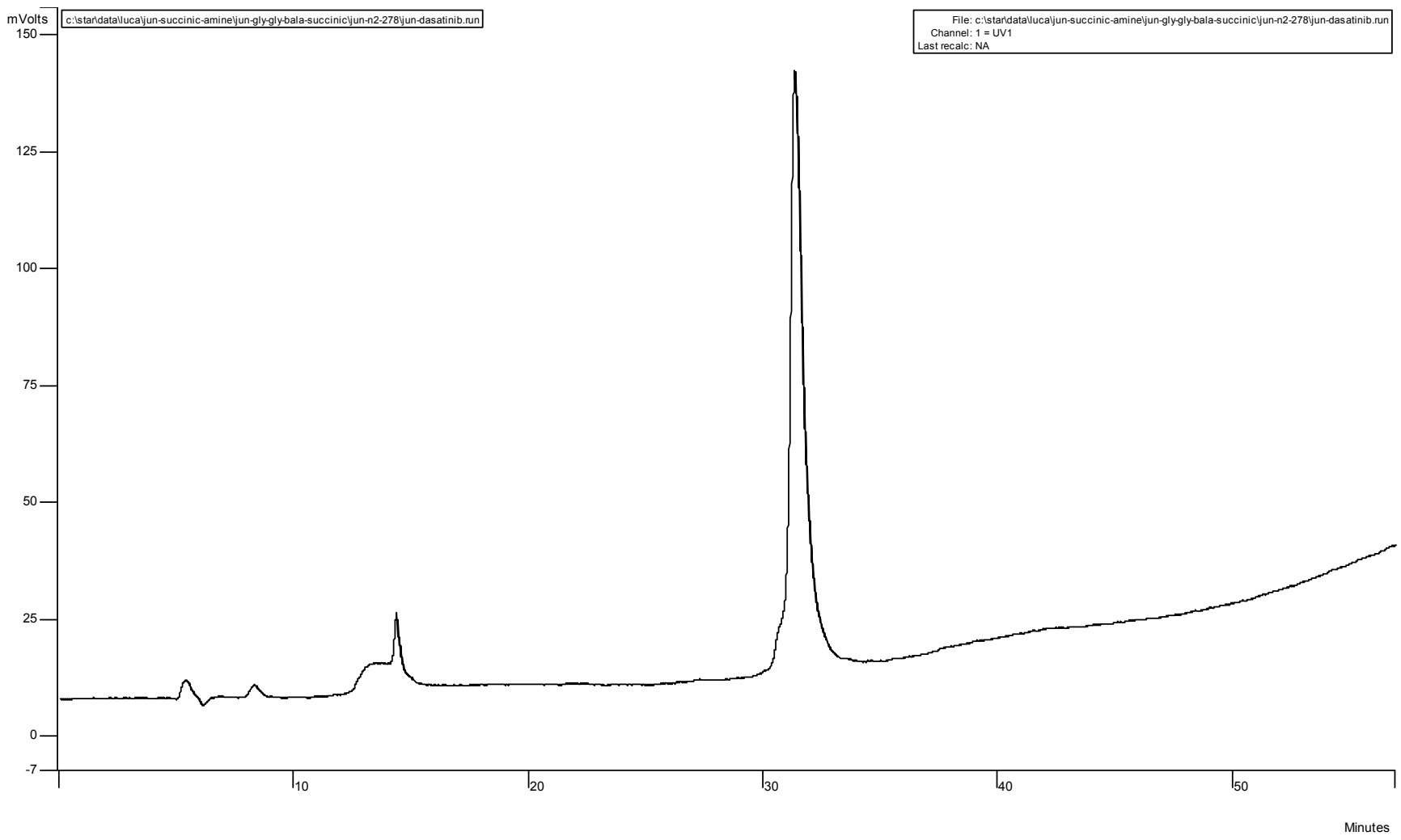

Figure S3 HPLC trace of compound **2**.

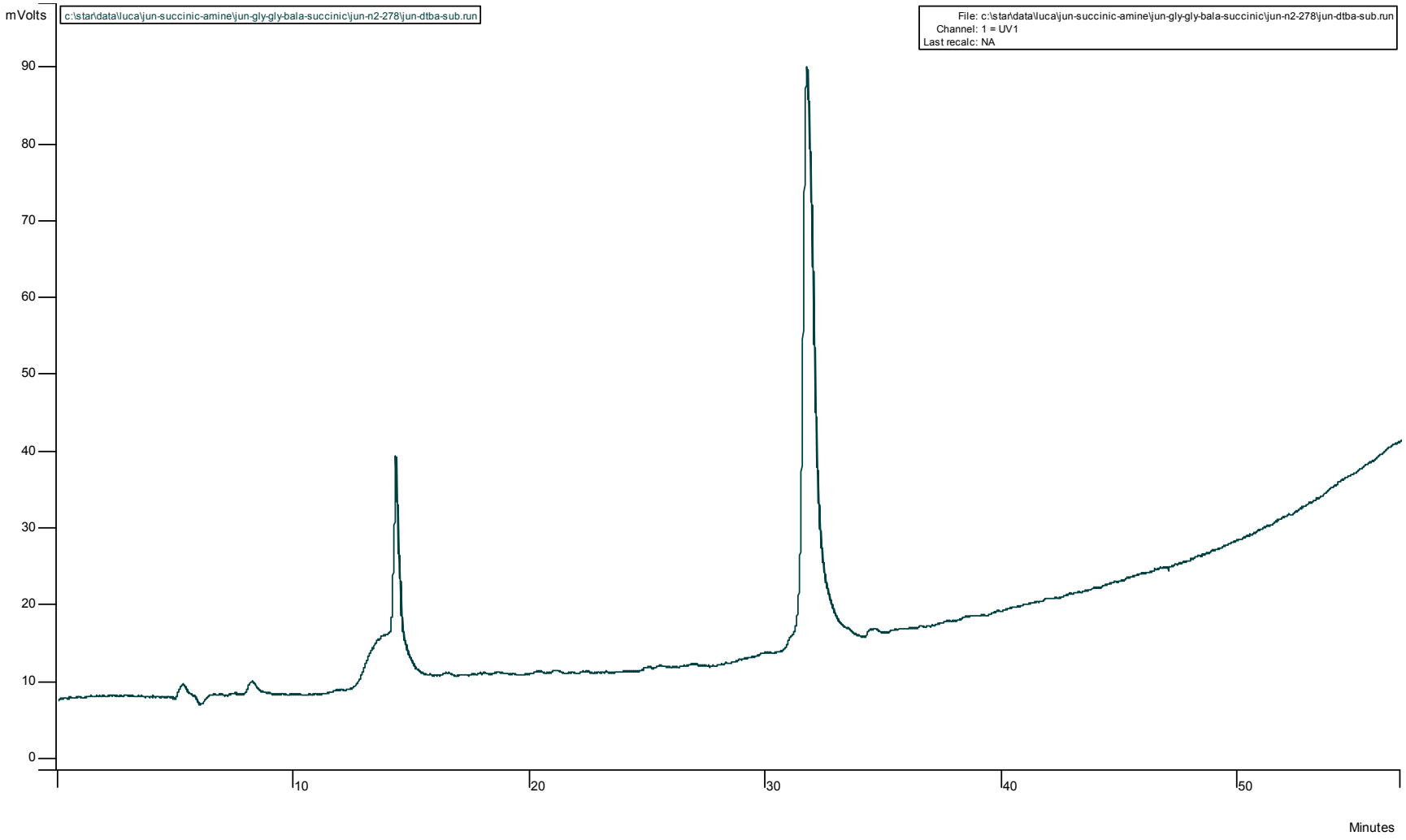

Figure S4 HPLC trace of compound **5.**

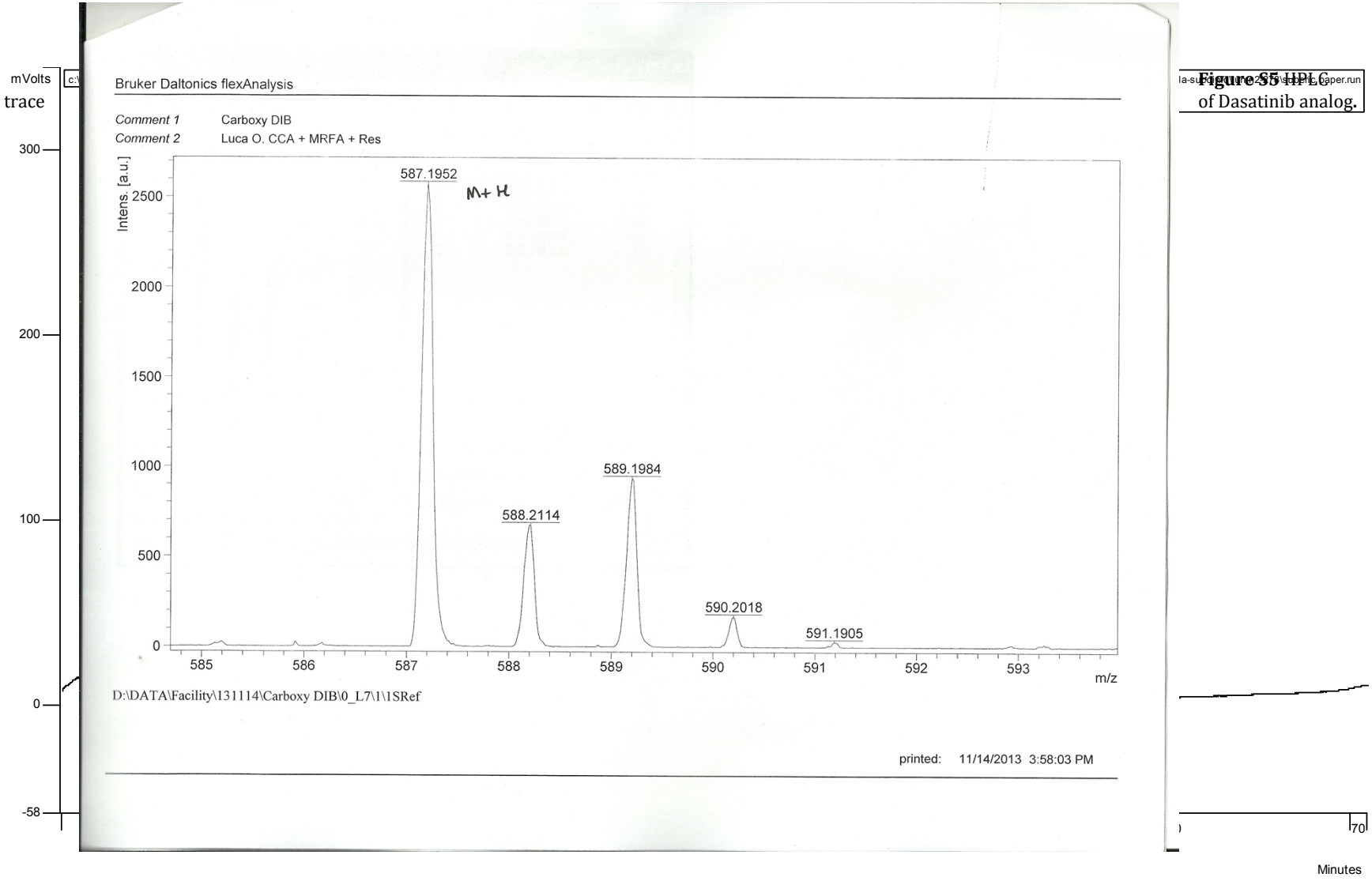

Figure S5 HPLC of Dasatinib analog.

Figure S6 carboxy-Dasatinib mass spectra

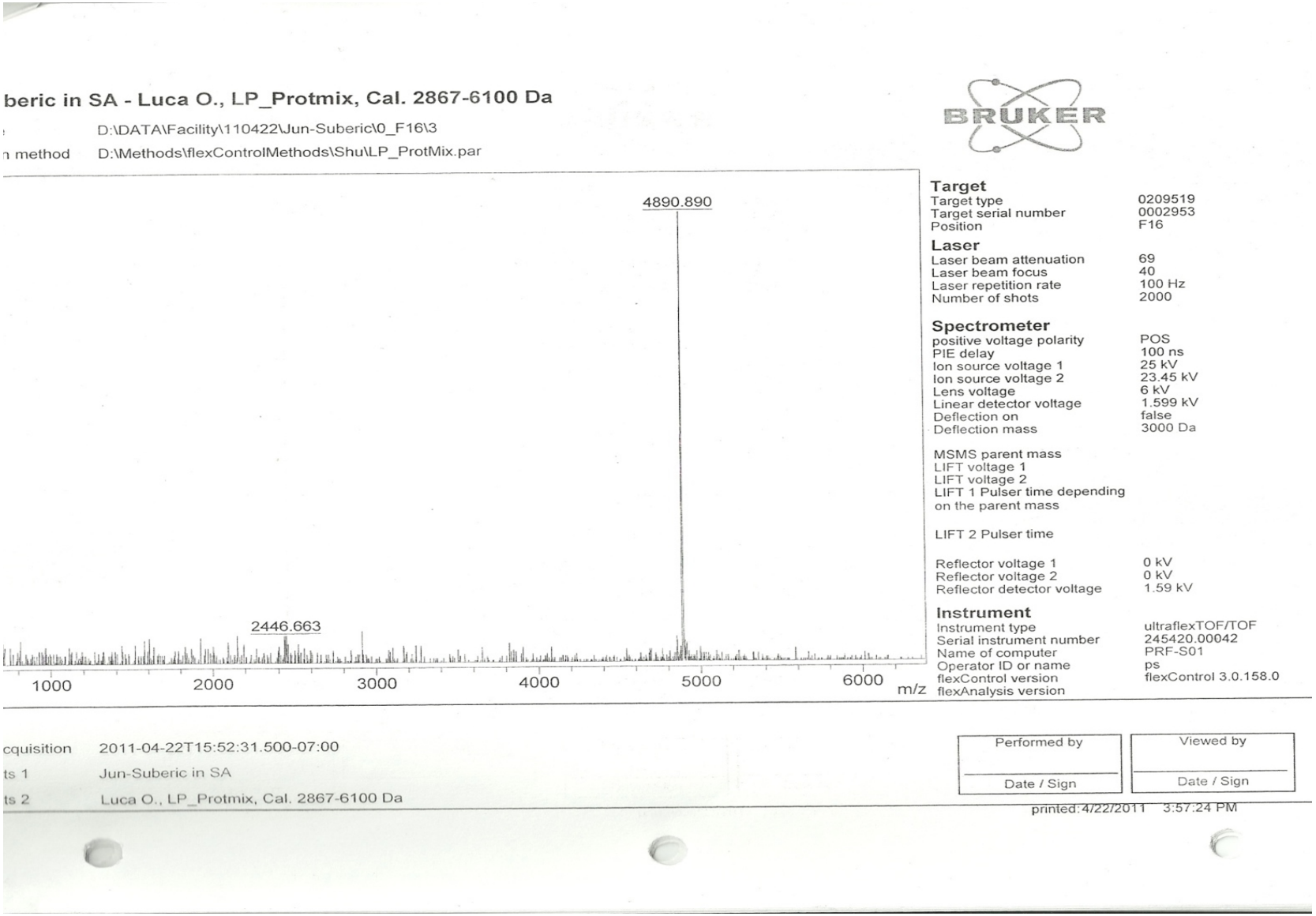

Figure S7 Compund **5** mass spectra

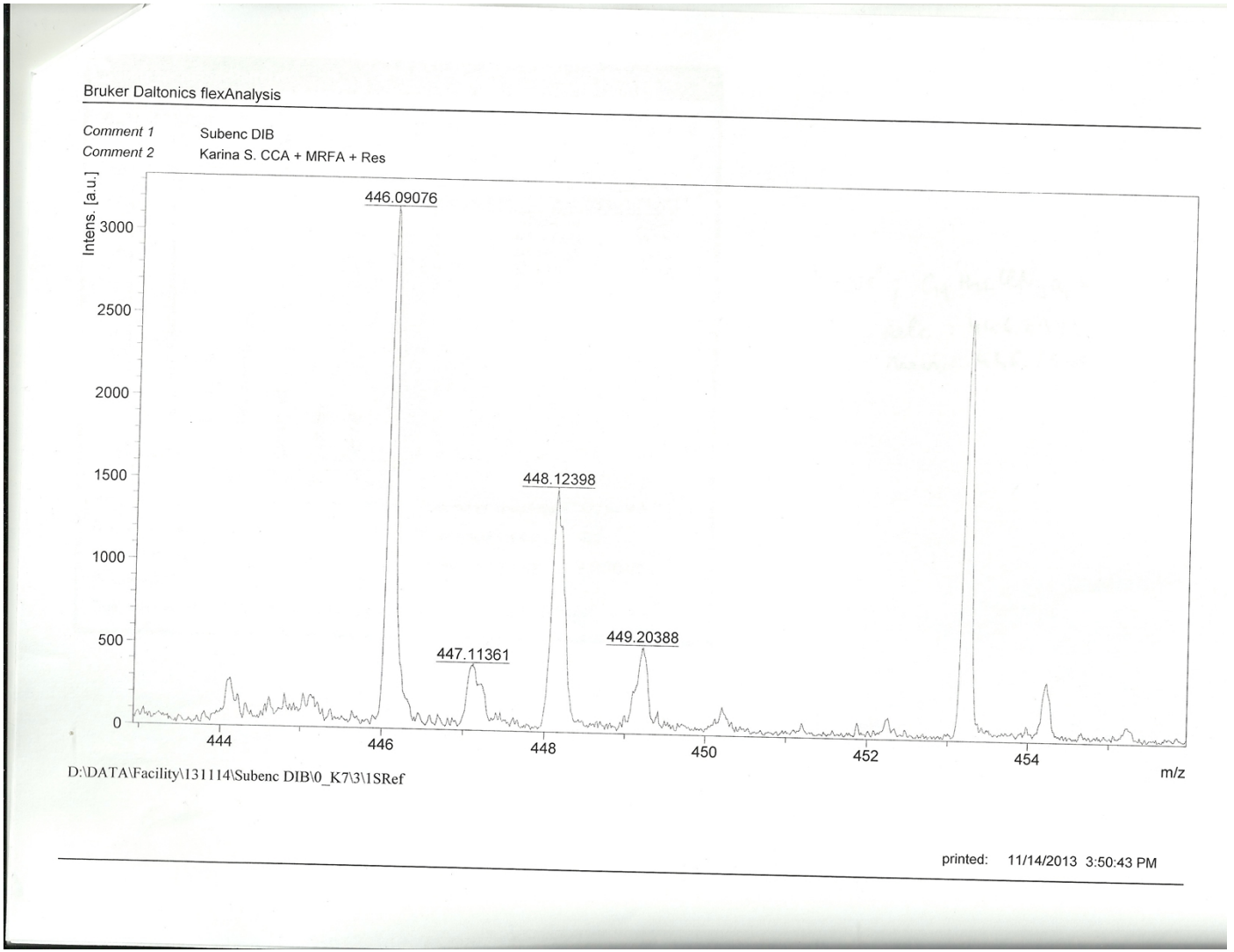

Figure S8 Dasatinib analog mass spectra

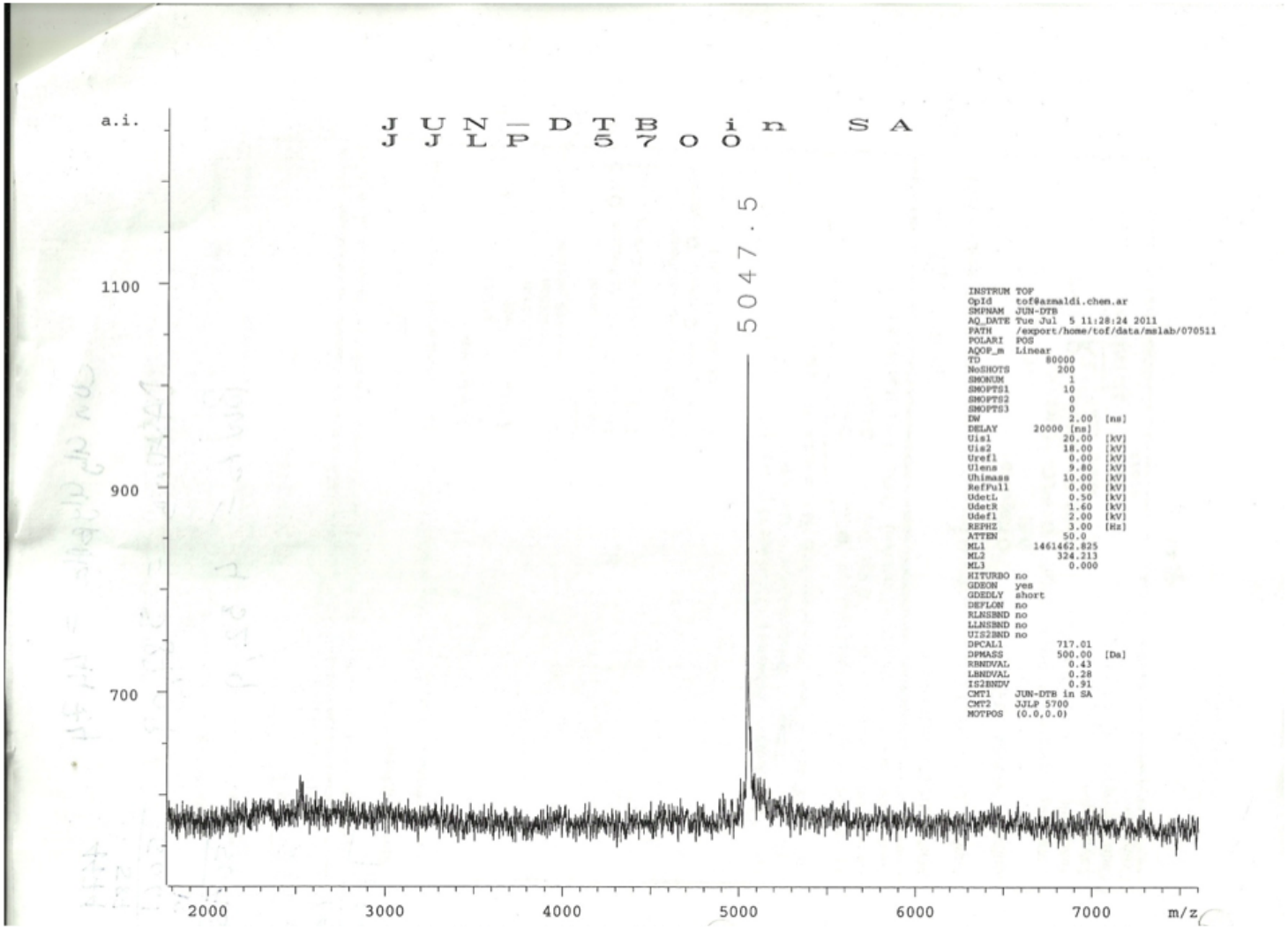

Figure S9 Compound **2** mass spectra

References

1. B. W. Jester, K. J. Cox, A. Gaj, C. D. Shomin, J. R. Porter and I. Ghosh, *J Am Chem Soc*, 2010, **132**, 11727-11735.
2. B. W. Jester, A. Gaj, C. D. Shomin, K. J. Cox and I. Ghosh, *J Med Chem*, 2012, **55**, 1526-1537.
3. J. Das, P. Chen, D. Norris, R. Padmanabha, J. Lin, R. V. Moquin, Z. Q. Shen, L. S. Cook, A. M. Doweyko, S. Pitt, S. H. Pang, D. R. Shen, Q. Fang, H. F. de Fex, K. W. McIntyre, D. J. Shuster, K. M. Gillooly, K. Behnia, G. L. Schieven, J. Wityak and J. C. Barrish, *J Med Chem*, 2006, **49**, 6819-6832.