Supplemental Data

Small Molecule Recognition of c-Src via the Imatinib-Binding Conformation

Arvin C. Dar, Michael S. Lopez, and Kevan M. Shokat

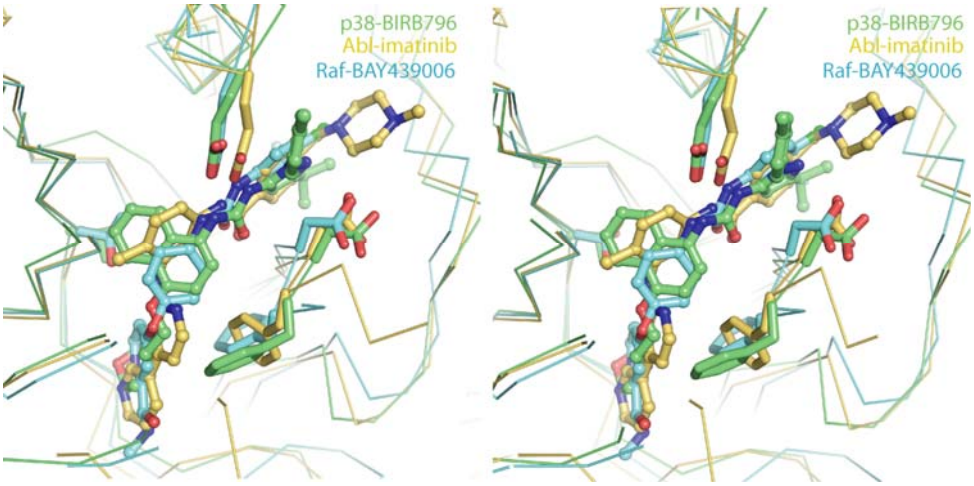

Figure S1. Three Different Type II Inhibitors Follow a Near Identical Path Within the Active Site of Three Different Kinases
This stereo figure depicts Abl in complex with Imatinib (yellow; PDB ID 2OIQ), bRaf in complex with BAY439006 (blue; PDB ID 1uwh), and p38 in complex with BIRB796 (yellow; PDB ID 1KV2). The structures were aligned based on the protein backbone atoms.

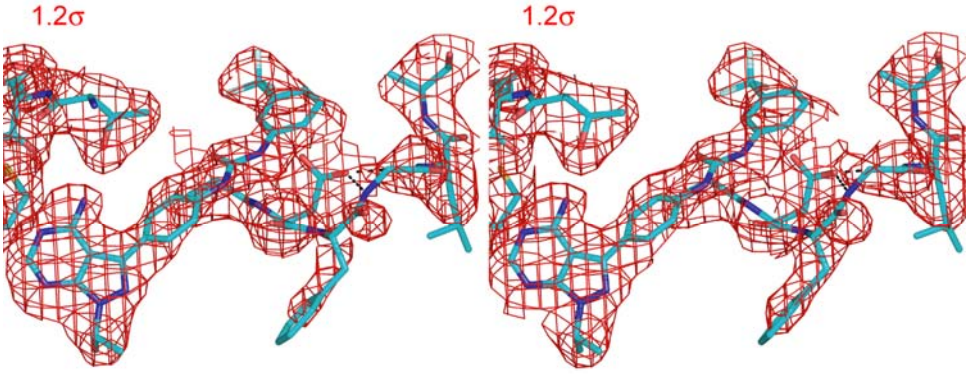

Figure S2. A Composite $|2F_o-F_c|$ Simulated Annealing Omit Electron Density Map (Bhat, 1988), Computed at 2.3 Å and Contoured at 1.2σ
The map is centered on compound **5**.

Table S1. Data Collection and Refinement Statistics

Data Collection		
Structure	Src-**3**	Src-**5**
Space Group	$P2_1$	P1
Unit Cell Dimensions	a=42.4Å, b=63.1Å, c=56.1Å α=90.0°, β=91.9°, γ=90.0°	a=42.4Å, b=63.7Å, c=73.8Å α=101.0°, β=90.2°, γ=90.1°
Numbers of protein molecules/asymmetric unit	1	2
X-ray Source	ALS 5.0.1	ALS 5.0.3
Wavelength (Å)	0.9774	0.9774
Resolution (Å)	30-2.80	50-2.30
Total Reflections	42531	117,900
Unique Reflections	7,350	31,919
I/σ	11.97(4.55)	12.50(2.79)
Completeness (%)	99.4(99.1)	96.1(82.1)
Rsym (%)	10.6(31.6)	8.5(31.2)
Model Refinement		
Resolution (Å)	30-2.8	50-2.3
Number of Reflections Rwork/Rfree	6970/366	29,310/1535
Rwork/Rfree	22.0/28.9	22.1/26.5
Rmsd from ideality in Bond length (Å)	0.007	0.007
Rmsd from ideality in Angles (°)	1.3	1.3
Number of Protein Atoms In Model	2036	4180
Number of Drug atoms In Model	36	33
Number of waters	59	176
Favored/Allowed/Outliers in the Ramachandran Plot (%)	92.2/7.8/0.0	96.1/3.7/0.2

Numbers in parentheses refer to the outer shell (2.80Å - 2.86Å) for Src-**3** and (2.30Å - 2.38Å) for Src-**5**

Supplemental Methods

Protein Expression and Purification

6xHIS fusions of c-Src or Abl were expressed in bacteria in the presence of the YopH phosphatase and GroEL chaperone based on a recently developed strategy (Seeliger et al., 2005). Briefly each kinase was purified in batch by Ni-NTA immobilized metal affinity chromatography. The 6X His was removed by TEV cleavage to yield the liberated kinase domain. Following cleavage, ion exchange chromatography was utilized to remove excess TEV and minor contaminants. In the final step the proteins were applied to a gel filtration column in 100 mM NaCl, 20 mM Tris, 5% glycerol, 2 mM DTT. Pooled fractions were concentrated and flash frozen in liquid nitrogen for storage. Proteins were isolated in their unphosphorylated state as revealed by western blot analysis (data not shown). Typical yields for either protein construct ranged from 1-10 mg of protein per 1L of bacterial culture.

In Vitro Kinase Assays

Purified c-Src or Abl were diluted in kinase reaction buffer (10mM HEPES [pH 7.2], 10 mM MgCl2, 0.2 mM DTT) to a concentration of approximately 10 nM and pre-incubated with 1mg/mL BSA, 2.5% (v/v) DMSO, 133 uM peptide (sequence EAIYAAPFKKK for Abl and EIYGEFKKK for c-Src), and varying concentrations of inhibitor. Kinase reactions were initiated by the addition of 100 μM cold ATP supplemented with 5 μCi γ^{32}P ATP and allowed to proceed at RT. At 10 minutes 1 μL of the reactions were spotted onto phosphocellulose sheets (P81, Whatman) and subsequently soaked in wash buffer (1.0% (v/v) phosphoric acid). The sheets were washed five times in buffer, dried, and transferred radioactivity was measured by phosphorimaging using a Typhoon scanner (Molecular Dynamics). Radioactive counts were quantified using ImageQuant software, and titration data were fit to a sigmoidal dose response to derive IC50 values using the Prism software package. Dose responses were based on a 12 point inhibitor titration, using 1/3 dilutions starting from 100 μM. Experiments were completed 2-4 times to derive mean values.

Crystallization and Structure Determination

Prior to crystallization, purified c-Src was applied to a S200 gel filtration column. Pooled fractions were concentrated to 3-10mg/mL and mixed with equimolar amounts of **3** or **5** in 100 mM NaCl, 10 mM Tris [pH 7.8], 5% glycerol, 2 mM DTT, 4% DMSO. Hanging drops containing 1 uL of complexes were mixed with equal volume of well buffer containing 4% PEG 4K, 16% glycerol, 50 mM NaAc, 100 mM MES [pH 6.5] and grown at 14°C to yield both the c-Src-**3** and c-Src-**5** crystals. Crystals were cryoprotected in well buffer supplemented with 20% glycerol and flash frozen. Diffraction data were collected at -170°C. Data processing and reduction was carried out using HKL2000 (Otwinowski and Minor, 1997) for the c-Src-**5** complex and XDS (Kabsch, 1993) for the c-Src-**3** complex. Both structures were solved by molecular replacement using 1YOJ lacking the activation segment, helix αC, and any ligands as the search model in the program PHASER (Mccoy et al., 2007). Molecular replacement solutions were modified and refined with alternate cycles of manual fitting and building into |2Fo-Fc| and composite omit electron density maps using Coot (Emsley and Cowtan, 2004). Refinement of the structures was conducted using simulated annealing and maximum likelihood protocols using CNS (Brunger et al., 1998)and REFMAC (Murshudov et al., 1997). Topology and parameter files for the inhibitors were generated using PRODRG (Schuttelkopf and van Aalten, 2004). Data collection and refinement statistics are shown in supplementary Table 1. A representative composite omit simulated annealing electron density ($|2F_o-F_c|$) map from the Src-**5** complex is shown in supplementary figure 2. All structural figures were prepared with PYMOL (Delano and Lam, 2005). Structures have been deposited in the Protein Data Bank under ID codes 3EL7 (Src-**3**) and 3EL8 (Src-**5**).

Chemical Synthesis

Starting materials were purchased from Sigma-Aldrich. Reactions were monitored by thin layer chromatography (TLC) and compounds were characterized by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Compounds **1-4** were synthesized starting from 3-nitrophenyl acetic acid, and **5** starting from 4-nitrophenyl boronic acid based on an established routes for preparing pyrazolopyrimidines (Bishop et al., 1999; Bishop et al., 1998; Blethrow et al., 2004) with the following modifications:

Compound 1. The intermediate 2-(1-methoxy-2-(3-nitrophenyl)ethylidene)malononitrile (0.4g; 1.6mmol) was combined with methylhydrazine (0.09 mL; 1.6 mmol; Sigma-Aldrich) in 10mL of THF for 1 hour on a ice-bath. The product was concentrated in vacuo and recrystallized from MeOH to yield 3-(3-nitrobenzyl)-5-amino-1-methyl-1*H*-pyrazole-4-carbonitrile (ESI-MS m/z [M+H]+ found 258.1, calculated 258.09). The crystallized product (0.2g; 0.8mmol) was combined with formamide (1.5mL) and heated to 160°C overnight. H_20 was added to the cooled reaction and the precipitate was filtered and dried to yield 3-(3-nitrobenzyl)-1-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine

(ESI-MS m/z [M+H]+ found 285.1, calculated 285.1). This precipitated intermediate (0.09 g; 0.33mmol) was then mixed with excess Zinc dust, 5 mL THF, 0.4 mL HOAc for 12 hours under Argon at room temperature. Afterwards the reaction was filtered through celite, extacted with EtOAc and concentrated in vacuo to yield 3-(3-aminobenzyl)-1-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 255.3, calculated 255.13).). To the reduced precursor, molar equivalents of 3-trifluoromethyl isocyanate (Sigma-Aldrich) were added drop wise in ice-cold CH_2Cl_2. The reaction proceeded until completion as judged by TLC, was concentrated in vacuo, resuspended in 50:50 H_20-CH_3CN, and purified on a C18 column in CH_3CN/H_20/0.1%TFA (1-100% gradient) to yield final compound **1** 1-(3-((4-amino-1-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)methyl)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (ESI-MS m/z [M+H]+ found 442.1, calculated 442.15; ^{1}H NMR (400 MHz, DMSO): δ 9.31 (s, 1H), 9.05 (s, 1H), 8.43 (s, 1H), 8.03 (s, 1H), 7.56 (d, *J* = 8Hz, 1H), 7.50 (t, *J* = 8Hz, 1H), 7.37 (s, 1H), 7.36 (d, *J* = 8HZ, 1H), 7.30 (d, *J* = 8Hz, 1H), 7.21 (t, *J* = 8Hz, 1H), 6.88 (d, *J* = 8Hz, 1H), 4.40 (s, 2H), 3.93 (s, 1H). ^{13}C NMR (400 MHz, DMSO): δ 33.48, 34.34, 98.15, 116.47 (d), 115.05, 117.11, 117.98, 119.13, 122.29, 122.91, 124.73 (q), 129.33, 130.00 (q), 130.32, 139.24, 140.15, 141.21, 146.00, 149.49, 152.37, 153.00, 153.76, 159.48 (q))

Compound 2. The intermediate 2-(1-methoxy-2-(3-nitrophenyl)ethylidene)malononitrile (1.2g; 4.7 mmol) was combined with isopropylhydrazine-HCl (0.57g; 5.2 mmol; Sigma-Aldrich), 1.4 mL triethylamine in 50mL EtOH for at 2 hours at RT. The reaction was concentrated in vacuo, resuspended in brine and extracted with chloroform. The organic layer was dried over $MgSO_4$. Following, the organic suspension was filtered, concentrated in vacuo, and purified on silica gel in 1% MeOH:$CHCl_3$ to yield 3-(3-nitrobenzyl)-5-amino-1-isopropyl-1*H*-pyrazole-4-carbonitrile (ESI-MS m/z [M+H]+ found 286.4, calculated 286.12). The product was combined with formamide as per compound **1** to yield 3-(3-nitrobenzyl)-1-isopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 313.4, calculated 313.13). Reduction of this material was completed as per compound **1** to yield 3-(3-aminobenzyl)-1-isopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine ESI-MS m/z [M+H]+ found 283.11, calculated 282.16. The reduced precursor was coupled to 3-trifluoromethyl isocyanate and purified as described for compound **1** to yield final compound **2** 1-(3-((4-amino-1-isopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)methyl)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (ESI-MS m/z [M+H]+ found 470.5, calculated 470.18; ^{1}H NMR (400 MHz, DMSO): δ 9.25 (1H, s), 8.97 (s, 1H), 8.36 (s, 1H), 8.05 (s, 1H), 7.48 – 7.54 (m, 2H), 7.41 (s, 1H), 7.30 (d, *J* = 8Hz, 2H), 7.20 (t, *J* = 8Hz, 1H0, 6.88 (d, *J* = 8Hz, 1H), 5.03 (septet, *J* = 8Hz, 1H), 1.48 (s, 6H). ^{13}C NMR (400 MHz, DMSO): δ 22.15, 33.44, 49.21, 98.32, 116.48 (d), 117.03, 118.92, 122.16, 122.67, 124.70(q), 129.33, 129.98 (q), 130.47 (q), 139.50, 140.07, 141.15, 145.31, 150.15, 151.77, 152.94, 154.32, 159.10 (q))

Compound 3: The intermediate 2-(1-methoxy-2-(3-nitrophenyl)ethylidene)malononitrile (0.3g; 1.3 mmol) was combined with hydrazine monohydrate (0.07 mL; 1.4 mmol; Sigma-Aldrich) in 5 mL EtOH for 1 hour at RT. The reaction was concentrated in vacuo to yield 3-(3-nitrobenzyl)-5-amino-1*H*-pyrazole-4-carbonitrile (0.3g; 1.3 mmol; ESI-MS m/z [M+H]+ found 244.5, calculated 244.1, ^{1}H NMR (400 MHz, DMSO): δ 11.77 (s, 1H), 8.09 (d, *J* = 8 Hz, 1H), 8.08 (s, 1H), 7.69 (d, *J* = 8 Hz, 1H), 7.60 (m, 1H), 6.33 (s, 2H), 3.96 (s, 2H)), which was subsequently combined with formamide (6mL) and heated to 180°C overnight. H_20 was added to the cooled reaction and the precipitate was filtered and dried to yield 3-(3-nitrobenzyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (0.26g; 0.96 mmol; ESI-MS m/z [M+H]+ found 271.4, calculated 271.09; ^{1}H NMR (400 MHz, DMSO): δ 8.18 (s, 1H), 8.10 (s, 1H), 8.06 (d, *J* = 8 Hz, 1H), 7.69 (d, *J* = 8Hz, 1H), 7.58 (m, 1H), 7.22 (br, 2H), 4.51 (s, 2H)). The recovered intermediate (0.05g; 0.18 mmol) was combined with bromocyclopentane (0.1 mL; 0.38 mmol), 0.125g K_2CO_3, in 1 mL DMF and refluxed under argon for 2 hours. The reaction was filtered to remove solid K_2CO_3, and the filtrate was combined with brine and the organic product was extracted in CH_2Cl_2 to yield 3-(3-nitrobenzyl)-1-cyclopentyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 339.5, calculated 339.15; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30 (s, 1H), 8.12 (m, 2H), 7.51 (m, 2H), 5.25 (pen, 8 Hz, 1H), 5.06 (br, 2H), 4.42 (s, 2H), 2.14 (m, 4H), 1.97 (m, 2H), 1.73 (m, 2H)). Reduction of this material was carried

out as per compound **1** to yield 3-(3-aminobenzyl)-1-cyclopentyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 309.5, calculated 309.17). The reduced precursor was coupled to 3-trifluoromethyl isocyanate and purified as described for compound **1** to yield final compound **3** 1-(3-((4-amino-1-cyclopentyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)methyl)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (ESI-MS m/z [M+H]+ found 496.4, calculated 496.2; ^{1}H NMR (400 MHz, DMSO): δ 9.22 (s, 1H), 8.93 (s, 1H), 8.34 (s, 1H), 8.06 (s, 1H), 7.50-7.60, (m, 2H), 7.43 (s, 1H), 7.18-7.22 (m, 3H), 6.90 (d, *J* = 8 Hz, 1H), 5.20 (pentet, *J* = 7 Hz, 1H), 4.40 (s, 2H), 1.98-2.13 (m, 4H), 1.85-1.95 (m, 2H), 1.62-1.73 (m, 2H); ^{13}C NMR (400 MHz, DMSO): δ 24.76, 32.33, 33.48, 57.62, 98.34, 114.48, 117.01, 118.17, 118.46, 118.89, 122.13, 122.34, 122.66, 129.97 (q), 139.53, 140.11, 141.16, 145.37, 150.60, 152.32, 152.94, 154.38, 159.00 (q)).

Compound 4: The intermediate 2-(1-methoxy-2-(3-nitrophenyl)ethylidene)malononitrile (1.2g; 4.7 mmol) was combined with tert-butylhydrazine-HCl (0.57g; 5.2 mmol; Sigma-Aldrich), 1.4 mL triethylamine in 50mL EtOH for at 2 hours at 80°C. The reaction was concentrated in vacuo, resuspended in brine and extracted with chloroform. The organic layer was dried over $MgSO_4$. Following, the organic suspension was filtered, concentrated in vacuo, and purified on silica gel in 1% MeOH:$CHCl_3$ to yield 3-(3-nitrobenzyl)-1-*tert*-butyl-5-amino-1*H*-pyrazole-4-carbonitrile (ESI-MS m/z [M-CH_3]+ found 285.5, calculated 285.14; ^{1}H NMR (400 MHz, DMSO): δ 8.11 (s, 1H), 8.08-8.12 (m, 1H), 7.69 (d, *J* = 8 Hz, 1H), 7.59-7.64 (m, 1H), 3.96 (s, 2H), 3.34 (br, 2H), 1.52 (s, 9H)). This product was combined with formamide as per compound **1** to yield 3-(3-nitrobenzyl)-1-*tert*-butyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 327.4, calculated 327.15; ^{1}H NMR (400 MHz, DMSO): δ 8.22 (s, 1H), 8.14 (s, 1H), 8.07 (d, *J* = 8 Hz, 1H), 7.67 (d, *J* = 8Hz, 1H), 7.59 (d, *J* = 8Hz, 1H), 7.22 (br, 2H), 4.52 (s, 2H), 1.70 (s, 9H)). Reduction of this material was completed as per compound **1** to yield 3-(3-aminobenzyl)-1-*tert*-butyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 297.13, calculated 297.17). The reduced precursor was coupled to 3-trifluoromethyl isocyanate and purified as described for compound **1** to yield final compound **4** 1-(3-((1-*tert*-butyl-4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)methyl)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (ESI-MS m/z [M+H]+ found 484.5, calculated 484.2; ^{1}H NMR (400 MHz, DMSO): δ 9.25 (s, 1H), 8.96 (s, 1H), 8.32 (s, 1H), 8.06 (s, 1H), 7.46-7.55 (m, 2H), 7.40 (s, 1H), 7.29 (d, *J* = 8Hz, 2H), 7.20 (t, *J* = 8Hz, 1H), 6.89 (d, *J* = 8Hz, 1H), 4.39 (s, 2H), 1.73 (s, 9H). ^{13}C NMR (400 MHz, DMSO): δ 29.25, 33.40, 60.74, 99.58, 116.43 (d), 116.95, 118.86, 122.13, 122.64, 124.69 (q), 129.29, 129.98 (q), 130.32, 139.60, 140.06, 141.16, 143.31, 149.70, 152.69, 152.92, 154.81, 159.12 (q)).

Compound 5: 4-nitrophenyl boronic acid (100 mg, 0.330 mmol; Sigma-Aldrich), was coupled to 3-iodo-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (140mg, 0.8248mmol) via the Suzuki reaction in 6 mL 1,2 methoxy ethane, 1 mL of saturated sodium carbonate, 1.65 mL EtOH, and 200 mg of polymer-bound tetrakis Palladium. The reaction was stirred under argon for 12 hours at room temperature, filtered through whatman paper to remove Palladium, mixed with brine, extracted in chloroform and the product was subsequently purified on silica in EtOAc and concentrated *in vacuo*. The purified solid 1-isopropyl-3-(4-nitrophenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 299.1, calculated 299.1; 100mg, 0.336 mmol) was combined with Zinc dust, 5 mL THF, 0.4 mL HOAc for 12 hours at room temperature. The reaction was filtered through celite, extacted with EtOAc and concentrated in vacuo to yield 3-(4-aminophenyl)-1-isopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (ESI-MS m/z [M+H]+ found 269.1, calculated 269.1). To the reduced product, molar equivalents of 3-trifluoromethyl isocyanate (Sigma-Aldrich) were added dropwise in ice-cold CH_2Cl_2. The reaction proceeded until completion as judged by TLC, was concentrated in vacuo, resuspended in 50:50 H_20-CH_3CN, and purified on a C18 column in CH_3CN/H_20/0.1%TFA (1-100% gradient) to yield **5** 1-(4-(4-amino-1-isopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (ESI-MS m/z [M+H]+ found 455.2, calculated 455.2; ^{1}H NMR (400 MHz, DMSO): δ 9.48 (s, 1H),

9.42 (s, 1H), 8.39 (s, 1H), 8.07 (s, 1H), 7.70 (d, *J* = 8Hz, 2H), 7.60 (d, *J* = 8Hz, 2H), 7.60 – 7.64 (m, 1H), 7.53 (t, *J* = 8Hz, 1H), 7.33 (d, *J* = 8Hz, 1H), 5.10 (septet, *J* = 6.8Hz, 1H), 1.51 (d, *J* = 6Hz, 6H), 3.10 (q, *J* = 4Hz, 1.5H, trace triethylamine), 1.18 (t, *J* = 8Hz, 2H, trace triethylamine).^{13}C NMR (400 MHz, DMSO): δ 9.08 (trace triethylamine), 22.23, 46.20 (trace triethylamine), 49.17, 97.40, 115.45, 116.0 (d), 119.20, 122.34, 124.70(q), 126.19, 129.35, 130.00 (q), 130.40, 140.85, 141.09, 145.20, 151.70, 152.35, 153.00, 155.72, 159.41 (q)).

Supplemental References

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