Electronic Supplementary Material (ESI) for ChemComm.

Degrasyn exhibits antibiotic activity against multi-resistant *Staphylococcus aureus* by modifying several essential cysteines

Kyu Myung Lee,[a] Philipp Le,[a] Stephan A. Sieber[a,] and Stephan M. Hacker[b,]**

[a]*Center for Integrated Protein Science at the Department of Chemistry and Chair of Organic Chemistry II, Technische Universität München, Garching bei München, Germany.*

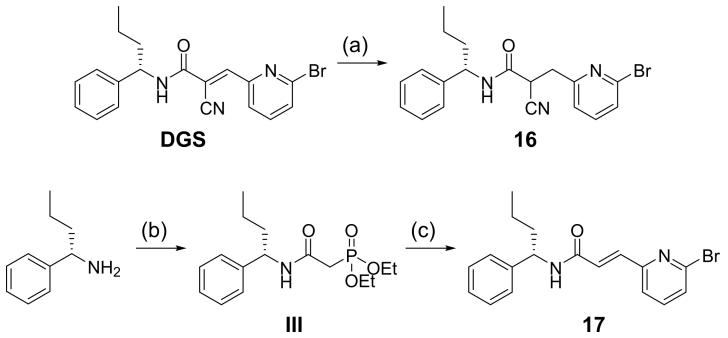
[b]*Department of Chemistry, Technische Universität München, Garching bei München, Germany.*

** E-Mail: stephan.m.hacker@tum.de and stephan.sieber@tum.de*

Content

1. Supplementary Scheme and Figures 3
2. Supporting Tables 8
3. Data Sets and Data Repository 9
4. Chemical Synthesis 13
4.1 General remarks 13
4.2 Synthesis of DGS and (*R*)-DGS 13
4.3 Synthesis of Derivatives of DGS 16
5. Biochemical Procedures 27
5.1 *S. aureus* strains and media 27
5.2 Overnight cultures of bacteria and cryostocks 27
5.3 Minimal inhibitory concentration (MIC) assay 27
5.4 Analytical gel-based ABPP 28
5.5 Conventional activity-based protein profiling (ABPP) 28
5.6 Competition experiment 31
5.7 Competitive isoDTB-ABPP 31
5.8 Global analysis of protein expression levels 36
6. Literature 38
7. NMR spectra 39

1. Supplementary Scheme and Figures



Scheme S1. Synthesis of **16** and **17** (a) $NaBH_4$, THF, 0 °C, 2h, 58%; (b) EDC.HCl, diethylphosphonoacetic acid, DMAP, DMF, 48h, 73%; (c) NaH, 6-Bromo-2-pyridinecarboxaldehyde, THF, 0 °C, 12h, 56%.

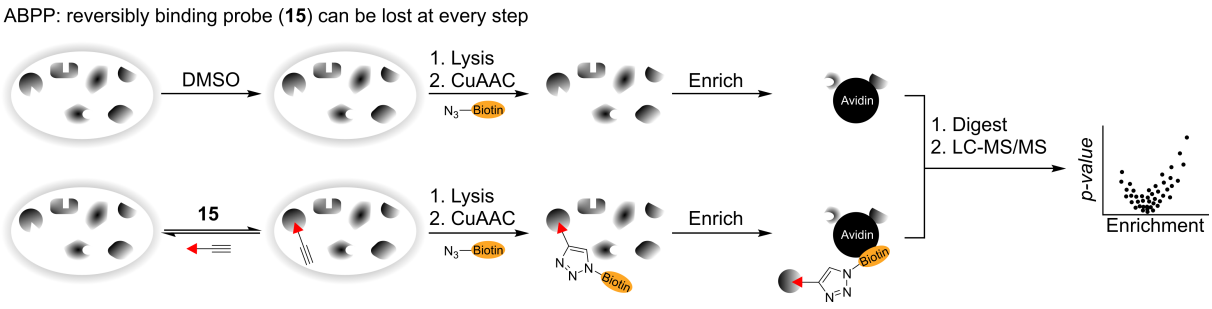


Figure S1. Workflow for conventional ABPP experiments. Enrichment is only seen, if the reversibly binding **15** is attached to the protein throughout the entire workflow.

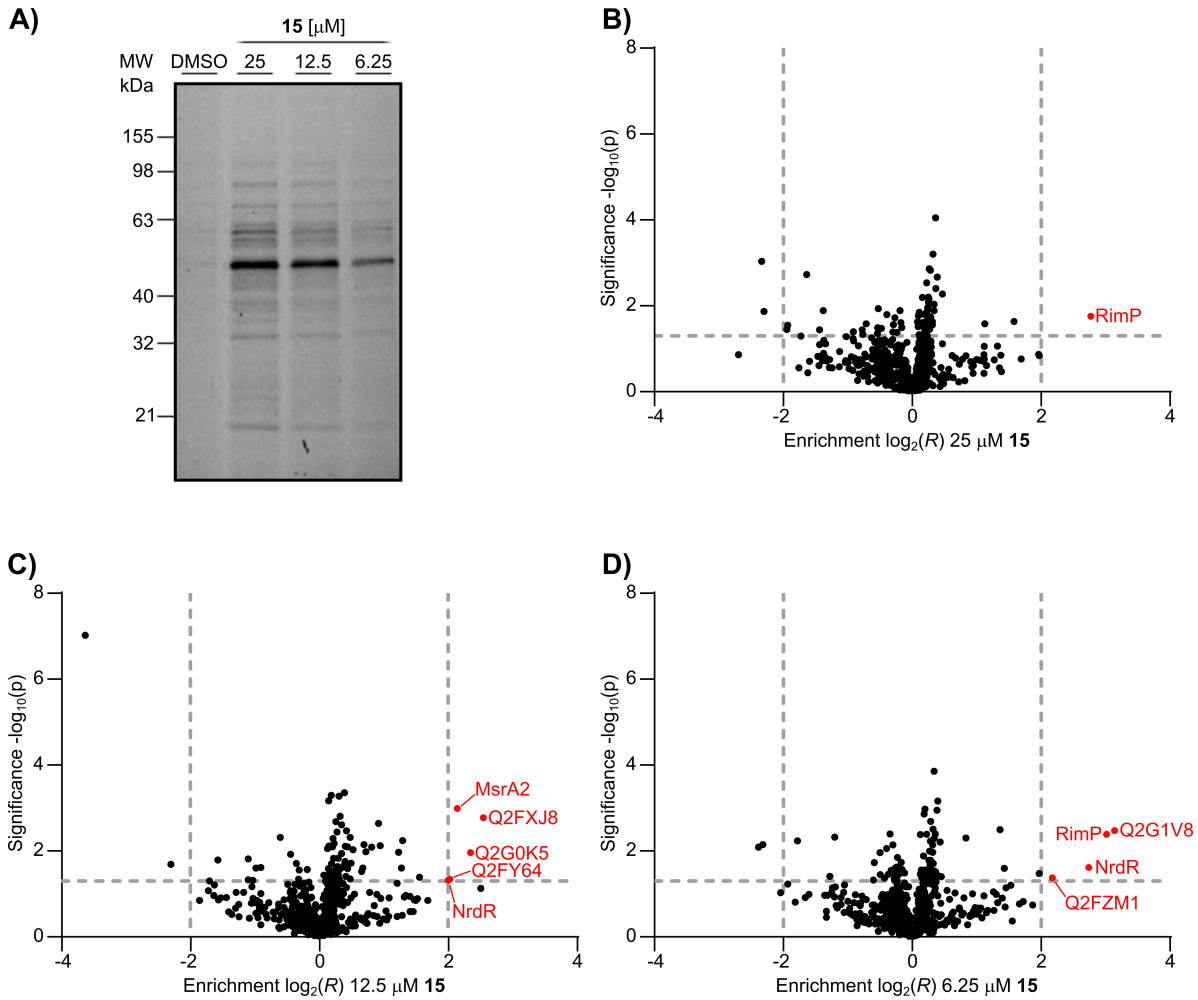


Figure S2. (**A**) Living *S. aureus* NCTC 8325 were treated with the **15** at the indicated concentration or DMSO as a control for 1 h at 37 °C. After lysis, Rhodamine azide was attached using CuAAC and labeling was analyzed using gel electrophoresis with in-gel fluorescence scanning. (**B** – **D**) Results of enrichment studies using conventional ABPP experiments with label-free quantification using **15** in *S. aureus* NCTC8325. Cells were treated with 25 µM (**B**), 12.5 µM (**C**) or 6.25 µM (**D**) probe, respectively, and compared to control cells treated with DMSO. The volcano plots display the statistical significance (p) of protein enrichment levels as a function of protein enrichment ratios (*R*) comparing probe-treated to control cells. The grey lines indicate a cut-off at $-\log_{10}(p) = 1.3$ and $\log_2(R) = \pm 2$ used as a criterion for hit selection. All data results from four independent biological replicates. Proteins that meet these criteria are highlighted in red. For each indicated protein, the name or the UniProt code of the respective protein are given.

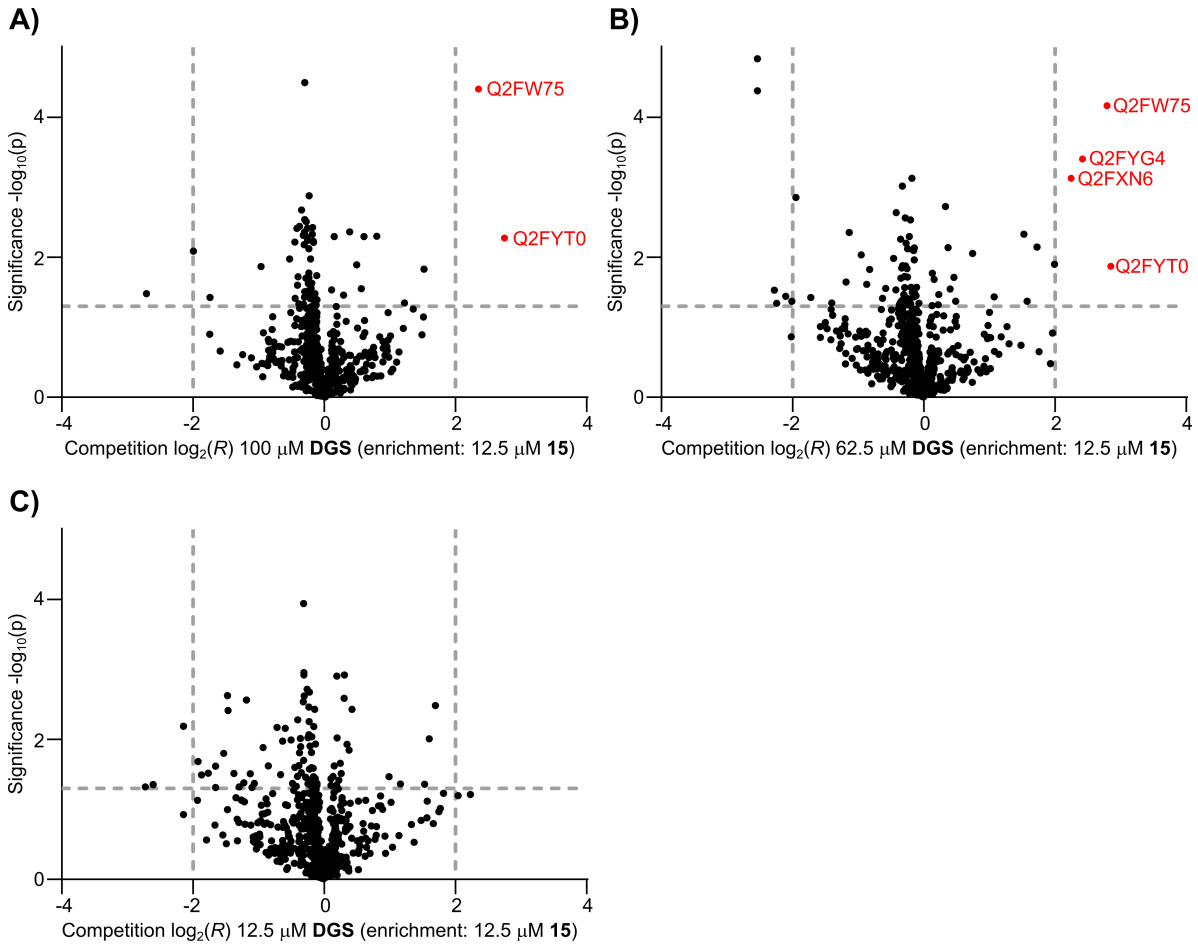


Figure S3. Results of competitive, conventional ABPP experiments using **DGS** and **15** in *S. aureus* NCTC8325. Cells were pre-treated with 100 µM (**A**), 62.5 µM (**B**) and 12.5 µM (**C**) **DGS**, respectively, and compared to cells pre-treated with DMSO. All samples were treated with 12.5 µM **15**. The volcano plots display the statistical significance (p) of protein enrichment levels as a function of protein enrichment ratios (*R*) comparing **DGS**-treated to control cells. The grey lines indicate a cut-off at $-\log_{10}(p) = 1.3$ and $\log_2(R) = \pm 2$ used as a criterion for hit selection. All data results from four independent biological replicates. Proteins that meet these criteria are highlighted in red. For each indicated protein, the name or the UniProt code of the respective protein are given.

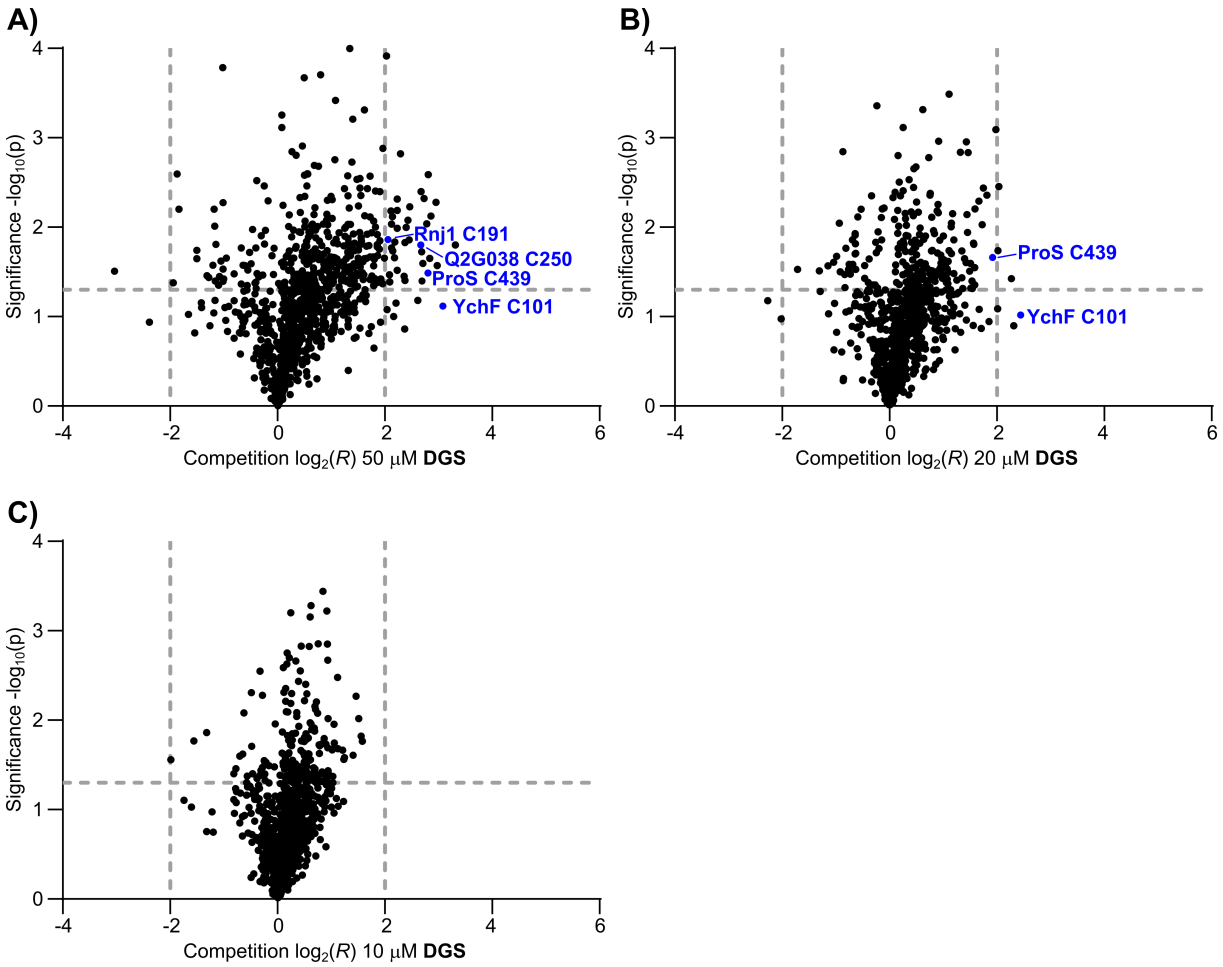


Figure S4. Volcano plots for the isoDTB-ABPP experiment with 50 µM (**A**), 20 µM (**B**) or 10 µM (**C**) **DGS** used as the competitor. Selected cysteines that are included in the concentration-dependent analysis (Figure 2C) are highlighted in blue. The grey lines indicate cut-offs at $-\log_{10}(p) = 1.3$ and $\log_2(R) = \pm 2$ that were used as a criterion for hit selection. For each indicated cysteine, the name or the UniProt code of the respective protein and the residue number of the competed cysteine are given. All data results from duplicates.

2. Supporting Tables

Table S1. All mass spectrometric data for the conventional ABPP studies, isoDTB-ABPP studies and global analysis of protein expression levels. This data can be found as additional data file "Table S1.xlsx" accompanying this manuscript.

Table S2. EC_{50} values with 95% confidence intervals for all cysteines shown in Figure 2C. Protein name and residue number: name of the protein according to UniProt and residue number of the modified cysteine, if available; Identifier: UniProt code of the protein and residue number of the modified cysteine; EC_{50} in µM: EC_{50} in µM for the concentration-dependent experiments using the isoDTB-ABPP technology; 95% Confidence interval EC_{50} in µM: 95% Confidence interval EC_{50} in µM for the concentration-dependent experiments using the isoDTB-ABPP technology; R^2: R^2 for the fit for the concentration-dependent experiments using the isoDTB-ABPP technology plotting $\log_{10}$(c(**DGS**)/µM) against % Competition using a non-linear dose-response curve fit with the function: % Competition = 100 - 100/(1+10^($\log_{10}$(c(**DGS**)/µM)-$\log_{10}$(EC_{50}))).

Protein name and residue number	Identifier	EC_{50} in µM	95% Confidence interval EC_{50} in µM	R^2
YchF C101	Q2G115 C101	5.22	3.38 to 7.51	0.89
ProS C439	Q2G1Z4 C439	8.27	5.65 to 11.64	0.93
	Q2G038 C250	9.84	7.70 to 12.39	0.98
	Q2G112 C97	14.93	8.96 to 23.95	0.94
Rnj1 C191	Q2FZG9 C191	17.55	15.49 to 19.85	0.99
GlmU C100	Q2G0S3 C100	22.32	18.90 to 26.32	0.99
	Q2FVV9 C390	25.57	24.25 to 26.96	1.00
TarJ C38	Q2G1B9 C38	33.35	18.59 to 60.16	0.93
FabF C165	Q2FZR9 C165	35.31	11.76 to 109.3	0.83
	Q2FY88 C137	52.72	45.09 to 61.79	0.99
	Q2FZF6 C123	79.21	72.74 to 86.38	1.00
Upp C159	Q2FWE6 C159	100.10	88.64 to 113.5	0.99
FusA C473	Q2G0N1 C473	124.20	99.74 to 157.3	0.98

3. Data Sets and Data Repository

Table S3. All samples analyzed by proteomics in this study.

Name of the data file*	Description of the sample
DGSp_25uM_1.raw	Enrichment analysis using conventional ABPP with 25 μM **15**, replicate 1
DGSp_25uM_2.raw	Enrichment analysis using conventional ABPP with 25 μM **15**, replicate 2
DGSp_25uM_3.raw	Enrichment analysis using conventional ABPP with 25 μM **15**, replicate 3
DGSp_25uM_4.raw	Enrichment analysis using conventional ABPP with 25 μM **15**, replicate 4
DGSp_12_5uM_1.raw	Enrichment analysis using conventional ABPP with 12.5 μM **15**, replicate 1
DGSp_12_5uM_2.raw	Enrichment analysis using conventional ABPP with 12.5 μM **15**, replicate 2
DGSp_12_5uM_3.raw	Enrichment analysis using conventional ABPP with 12.5 μM **15**, replicate 3
DGSp_12_5uM_4.raw	Enrichment analysis using conventional ABPP with 12.5 μM **15**, replicate 4
DGSp_6_25uM_1.raw	Enrichment analysis using conventional ABPP with 6.25 μM **15**, replicate 1
DGSp_6_25uM_2.raw	Enrichment analysis using conventional ABPP with 6.25 μM **15**, replicate 2
DGSp_6_25uM_3.raw	Enrichment analysis using conventional ABPP with 6.25 μM **15**, replicate 3
DGSp_6_25uM_4.raw	Enrichment analysis using conventional ABPP with 6.25 μM **15**, replicate 4
DGSp_DMSO_1.raw	Enrichment analysis using conventional ABPP with DMSO as a control, replicate 1
DGSp_DMSO_2.raw	Enrichment analysis using conventional ABPP with DMSO as a control, replicate 2
DGSp_DMSO_3.raw	Enrichment analysis using conventional ABPP with DMSO as a control, replicate 3
DGSp_DMSO_4.raw	Enrichment analysis using conventional ABPP with DMSO as a control, replicate 4
DGS_100uM_ DGSp_ 12_5uM_1.raw	Competition analysis using conventional ABPP with pretreatment with 100 μM **DGS** and enrichment with 12.5 μM **15**, replicate 1
DGS_100uM_ DGSp_ 12_5uM_2.raw	Competition analysis using conventional ABPP with pretreatment with 100 μM **DGS** and enrichment with 12.5 μM **15**, replicate 2
DGS_100uM_ DGSp_ 12_5uM_3.raw	Competition analysis using conventional ABPP with pretreatment with 100 μM **DGS** and enrichment with 12.5 μM **15**, replicate 3
DGS_100uM_ DGSp_ 12_5uM_4.raw	Competition analysis using conventional ABPP with pretreatment with 100 μM **DGS** and enrichment with 12.5 μM **15**, replicate 4
DGS_62_5uM_ DGSp_ 12_5uM_1.raw	Competition analysis using conventional ABPP with pretreatment with 62.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 1
DGS_62_5uM_ DGSp_ 12_5uM_2.raw	Competition analysis using conventional ABPP with pretreatment with 62.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 2
DGS_62_5uM_ DGSp_ 12_5uM_3.raw	Competition analysis using conventional ABPP with pretreatment with 62.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 3

DGS_62_5uM_ DGSp_ 12_5uM_4.raw	Competition analysis using conventional ABPP with pretreatment with 62.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 4
DGS_12_5uM_ DGSp_ 12_5uM_1.raw	Competition analysis using conventional ABPP with pretreatment with 12.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 1
DGS_12_5uM_ DGSp_ 12_5uM_2.raw	Competition analysis using conventional ABPP with pretreatment with 12.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 2
DGS_12_5uM_ DGSp_ 12_5uM_3.raw	Competition analysis using conventional ABPP with pretreatment with 12.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 3
DGS_12_5uM_ DGSp_ 12_5uM_4.raw	Competition analysis using conventional ABPP with pretreatment with 12.5 μM **DGS** and enrichment with 12.5 μM **15**, replicate 4
DMSO_DGSp_12_5uM_1.raw	Competition analysis using conventional ABPP with pretreatment with DMSo as a control and enrichment with 12.5 μM **15**, replicate 1
DMSO_DGSp_12_5uM_2.raw	Competition analysis using conventional ABPP with pretreatment with DMSo as a control and enrichment with 12.5 μM **15**, replicate 2
DMSO_DGSp_12_5uM_3.raw	Competition analysis using conventional ABPP with pretreatment with DMSo as a control and enrichment with 12.5 μM **15**, replicate 3
DMSO_DGSp_12_5uM_4.raw	Competition analysis using conventional ABPP with pretreatment with DMSo as a control and enrichment with 12.5 μM **15**, replicate 4
20180518_SMH_180518_P1.raw	Competition analysis using isoDTB- ABPP with pretreatment with 100 μM **DGS**, replicate 1
20180518_SMH_180518_P2.raw	Competition analysis using isoDTB- ABPP with pretreatment with 100 μM **DGS**, replicate 2
180918_SMH_180719_P17.raw	Competition analysis using isoDTB- ABPP with pretreatment with 50 μM **DGS**, replicate 1
180918_SMH_180719_P18.raw	Competition analysis using isoDTB- ABPP with pretreatment with 50 μM **DGS**, replicate 2
180918_SMH_180719_P19.raw	Competition analysis using isoDTB- ABPP with pretreatment with 20 μM **DGS**, replicate 1
180918_SMH_180719_P20.raw	Competition analysis using isoDTB- ABPP with pretreatment with 20 μM **DGS**, replicate 2
20180518_SMH_180518_P3.raw	Competition analysis using isoDTB- ABPP with pretreatment with 10 μM **DGS**, replicate 1
20180518_SMH_180518_P4.raw	Competition analysis using isoDTB- ABPP with pretreatment with 10 μM **DGS**, replicate 2
Global_analysis_DGS_1.raw	Global analysis of protein expression levels after treatment with 3.1 μM **DGS**, replicate 1
Global_analysis_DGS_2.raw	Global analysis of protein expression levels after treatment with 3.1 μM **DGS**, replicate 2
Global_analysis_DGS_3.raw	Global analysis of protein expression levels after treatment with 3.1 μM **DGS**, replicate 3
Global_analysis_DGS_4.raw	Global analysis of protein expression levels after treatment with 3.1 μM **DGS**, replicate 4
Global_analysis_DMSO_1.raw	Global analysis of protein expression levels after treatment with DMSO as a control, replicate 1
Global_analysis_DMSO_2.raw	Global analysis of protein expression levels after treatment with DMSO as a control, replicate 1
Global_analysis_DMSO_3.raw	Global analysis of protein expression levels after treatment with DMSO as a control, replicate 1

Global_analysis_DMSO_4.raw	Global analysis of protein expression levels after treatment with DMSO as a control, replicate 1

*DGSp indicates compound **15**.

Table S4. All different MaxQuant analyses performed in this study.

Name of the Analysis	Included Samples*
Enrichment_Conventional_ABPP	DGSp_25uM_1.raw DGSp_25uM_2.raw DGSp_25uM_3.raw DGSp_25uM_4.raw DGSp_12_5uM_1.raw DGSp_12_5uM_2.raw DGSp_12_5uM_3.raw DGSp_12_5uM_4.raw DGSp_6_25uM_1.raw DGSp_6_25uM_2.raw DGSp_6_25uM_3.raw DGSp_6_25uM_4.raw DGSp_DMSO_1.raw DGSp_DMSO_2.raw DGSp_DMSO_3.raw DGSp_DMSO_4.raw
Competition_Conventional_ABPP	DGS_100uM_ DGSp_ 12_5uM_1.raw DGS_100uM_ DGSp_ 12_5uM_2.raw DGS_100uM_ DGSp_ 12_5uM_3.raw DGS_100uM_ DGSp_ 12_5uM_4.raw DGS_62_5uM_ DGSp_ 12_5uM_1.raw DGS_62_5uM_ DGSp_ 12_5uM_2.raw DGS_62_5uM_ DGSp_ 12_5uM_3.raw DGS_62_5uM_ DGSp_ 12_5uM_4.raw DGS_12_5uM_ DGSp_ 12_5uM_1.raw DGS_12_5uM_ DGSp_ 12_5uM_2.raw DGS_12_5uM_ DGSp_ 12_5uM_3.raw DGS_12_5uM_ DGSp_ 12_5uM_4.raw DMSO_DGSp_12_5uM_1.raw DMSO_DGSp_12_5uM_2.raw DMSO_DGSp_12_5uM_3.raw DMSO_DGSp_12_5uM_4.raw
isoDTB_ABPP	20180518_SMH_180518_P1.raw 20180518_SMH_180518_P2.raw 180918_SMH_180719_P17.raw 180918_SMH_180719_P18.raw 180918_SMH_180719_P19.raw 180918_SMH_180719_P20.raw 20180518_SMH_180518_P3.raw 20180518_SMH_180518_P4.raw
Global_Analysis_of_Protein_Expression	Global_analysis_DGS_1.raw Global_analysis_DGS_2.raw Global_analysis_DGS_3.raw Global_analysis_DGS_4.raw Global_analysis_DMSO_1.raw Global_analysis_DMSO_2.raw Global_analysis_DMSO_3.raw Global_analysis_DMSO_4.raw

*DGSp indicates compound **15**.

All mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium[1] via the PRIDE partner repository[2] with the dataset identifier PXD016413. We have deposited all raw files, all FASTA databases ("SA1_Kyu_CU.fasta" for the isoDTB-ABPP studies, "FASTA_S_aureus_NCTC8325_KYU.fasta" for all other studies) and all analyses described here. For all analyses, there is a zip file with the name of the analysis that contains the "txt"-folder of this MaxQuant analysis.

4. Chemical Synthesis

4.1 General remarks

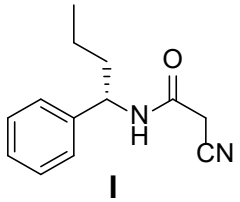
All air or water sensitive reactions were carried out under argon in oven-dried glassware. Chemicals and solvents were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics, TCI Europe and Merck, were of reagent grade or better and were used without further purification. In all reactions, temperatures were measured externally. Solvents removed under reduced pressure were evaporated at 40 °C. Flash column chromatography was performed on silica gel (40-63 μm) by VWR, elution solvents were distilled prior to use. Analytical thin-layer chromatography was carried out on aluminium-baked TLC Silica gel plates by Merck. Components were visualized by UV detection (λ=254 nm, 312 nm) or stained via aqueous $KMnO_4$ or aqueous cerium molybdate (Hanessian's stain). ^1H NMR and ^{13}C spectra of small molecules were recorded on Bruker instruments (300MHz, 400 MHz or 500 MHz) and referenced to the residual proton signal of the deuterated solvent. ($CDCl_3$, DMSO-d_6). Carbon samples were referenced externally against the residual ^{13}C signal of the solvent. Multiplets are described using following abbreviations: s - singlet, d – doublet, dd – doublet of doublet, t - triplet, q - quartet, and m - multiplet. HR-MS-ESI spectra were recorded with a Thermo Scientific LTQ FT Ultra.

Coupling reactions between amines and 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-3-oxopropanenitrile[3] were accomplished according to Ried *et al.*[4] An identical approach of the reaction was published by Gόmez *et al.*[5]

Based on NMR spectroscopy, the purity of all compounds used for biological testing was estimated to be over 95%.

4.2 Synthesis of DGS and (*R*)-DGS

(*S*)-2-Cyano-*N*-(1-phenylbutyl)acetamide (I)[3, 4]

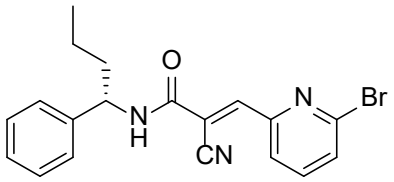


I

To a stirred solution of (*S*)-1-phenylbutan-1-amine (500 mg, 3.35 mmol) in toluene (30 mL) was added 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-3-oxopropanenitrile (547 mg, 1.34 mmol) and refluxed for 2 h. After cooling to room temperature, the mixture was evaporated, and purified

by column chromatography (40% EtOAc in hexane) to afford compound **I** as a white solid (540 mg, 75%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 7.31 – 7.18 (m, 5H), 6.30 (s, 1H), 4.85 (q, *J* = 7.6 Hz, 1H), 3.28 (d, *J* = 19.1 Hz, 1H), 3.22 (d, *J* = 19.1 Hz, 1H), 1.83 – 1.65 (m, 2H), 1.34 – 1.13 (m, 2H), 0.85 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, *CDCl$_3$*): 160.16, 141.29, 128.98, 127.94, 126.67, 114.92, 54.54, 38.10, 26.06, 19.55, 13.85. HRMS (ESI) calcd. for $C_{13}H_{16}N_2O$ $[M+H]^+$ 217.1335, found 217.1336.

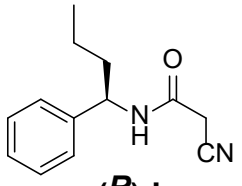
(*S,E*)-3-(6-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (DGS)[5]



DGS

To a solution of (*S*)-2-cyano-*N*-(1-phenylbutyl)acetamide (**I**, 100 mg, 0.462 mmol) in EtOH (4.5 mL) was added 6-bromo-2-pyridinecarboxaldehyde (95 mg, 0.51 mmol) and piperidine (9 μL, 0.09 mmol). The reaction mixture was refluxed for 3 h and then cooled to room temperature. The solvent was evaporated *in vacuo*, and the residue was purified by column chromatography (30% EtOAc in hexane) to afford **DGS** as a white solid (85 mg, 65%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 8.18 (s, 1H), 7.68 – 7.55 (m, 3H), 7.39 – 7.26 (m, 5H), 6.78 (d, *J* = 8.0 Hz, 1H), 5.08 (q, *J* = 7.6 Hz, 1H), 1.93 – 1.82 (m, 2H), 1.47 – 1.24 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*): 158.75, 151.03, 148.39, 142.60, 141.47, 139.27, 130.78, 128.97, 127.86, 126.65, 125.57, 115.95, 109.64, 54.79, 38.33, 19.59, 13.88. HRMS (ESI) calcd. for $C_{19}H_{18}BrN_3O$ $[M+H]^+$ 384.0706, found 384.0704.

(*R*)-2-Cyano-*N*-(1-phenylbutyl)acetamide ((*R*)-I)[3, 4]

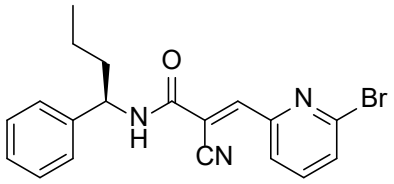


(*R*)-I

To a stirred solution of (*R*)-1-phenylbutan-1-amine (500 mg, 3.35 mmol) in toluene (30 mL) was added 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-3-oxopropanenitrile (547 mg, 1.34 mmol) and

refluxed for 2 h. After cooling to room temperature, the mixture was evaporated, and purified by column chromatography (40% EtOAc in hexane) to afford compound **(*R*)-I** as a white solid (570 mg, 79%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 7.31 – 7.18 (m, 5H), 6.30 (s, 1H), 4.85 (q, *J* = 7.6 Hz, 1H), 3.28 (d, *J* = 19.1 Hz, 1H), 3.22 (d, *J* = 19.1 Hz, 1H), 1.83 – 1.65 (m, 2H), 1.34 – 1.13 (m, 2H), 0.85 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, *CDCl$_3$*): 160.16, 141.29, 128.98, 127.94, 126.67, 114.92, 54.54, 38.10, 26.06, 19.55, 13.85. HRMS (ESI) calcd. for $C_{13}H_{16}N_2O$ $[M+H]^+$ 217.1335, found 217.1336.

(*R*,*E*)-3-(6-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide ((*R*)-DGS)[5]

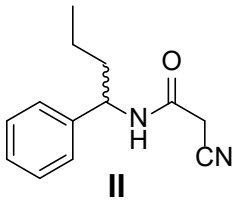


(*R*)-DGS

To a solution of (*R*)-2-cyano-*N*-(1-phenylbutyl)acetamide (**(*R*)-I**, 100 mg, 0.462 mmol) in EtOH (4.5 mL) was added 6-bromo-2-pyridinecarboxaldehyde (95 mg, 0.51 mmol) and piperidine (9 μL, 0.09 mmol). The reaction mixture was refluxed for 3 h and then cooled to room temperature. The solvent was evaporated *in vacuo*, and the residue was purified by column chromatography (30% EtOAc in hexane) to afford **(*R*)-DGS** as a white solid (114 mg, 63%).[5] ^{1}H NMR (300 MHz, *CDCl$_3$*): 8.18 (s, 1H), 7.68 – 7.55 (m, 3H), 7.39 – 7.26 (m, 5H), 6.78 (d, *J* = 8.0 Hz, 1H), 5.08 (q, *J* = 7.6 Hz, 1H), 1.93 – 1.82 (m, 2H), 1.47 – 1.24 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*): 158.75, 151.03, 148.39, 142.60, 141.47, 139.27, 130.78, 128.97, 127.86, 126.65, 125.57, 115.95, 109.64, 54.79, 38.33, 19.59, 13.88. HRMS (ESI) calcd. for $C_{19}H_{18}BrN_3O$ $[M+H]^+$ 384.0706, found 384.0706.

4.3 Synthesis of Derivatives of DGS

2-Cyano-*N*-(1-phenylbutyl)acetamide (II)[3, 4]



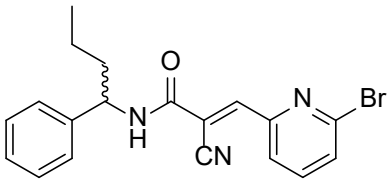
II

To a solution of 1-phenylbutan-1-one (5.00 g, 33.7 mmol) in EtOH (53 mL) was added hydroxylamine hydrochloride (4.69 g, 67.5 mmol) and sodium hydroxide (5.40 g, 135 mmol) in H_2O (17 mL). The mixture was refluxed for 3 h, cooled to room temperature and evaporated under vacuum. The aqueous phase was extracted with EtOAc, and the organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was dissolved in MeOH (100 mL) and hydrogenated with 1 bar H_2 using 10 wt% Pd/C catalyst (500 mg) at room temperature for 24 h. The reaction mixture was filtered through a pad of celite. The combined filtrate was concentrated *in vacuo* to provide the crude residue. Subsequently, to a stirred solution of the crude product in toluene (30 mL) was added 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-3-oxopropanenitrile (5.51 g, 33.7 mmol) and refluxed for 2 h. After cooling to room temperature, the mixture was evaporated and purified by column chromatography (40% EtOAc in hexane) to afford compound **II** as a white solid (5.02 g, 69%).[3, 4] ^{1}H NMR (300 MHz, *CDCl₃*): 7.31 – 7.18 (m, 5H), 6.30 (s, 1H), 4.85 (q, *J* = 7.6 Hz, 1H), 3.28 (d, *J* = 19.1 Hz, 1H), 3.22 (d, *J* = 19.1 Hz, 1H), 1.83 – 1.65 (m, 2H), 1.34 – 1.13 (m, 2H), 0.85 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, *CDCl₃*): 160.16, 141.29, 128.98, 127.94, 126.67, 114.92, 54.54, 38.10, 26.06, 19.55, 13.85. HRMS (ESI) calcd. for $C_{13}H_{16}N_2O$ $[M+H]^+$ 217.1335, found 217.1335.

General method for the condensation of 2-cyano-*N*-(1-phenylbutyl)acetamide (II) with aryl aldehyde.

To a solution of 2-cyano-*N*-(1-phenylbutyl)acetamide (**II**, 1 equiv.) in EtOH (0.1 M) was added the corresponding aryl aldehyde (1.1 equiv.) and piperidine (0.2 equiv.). The reaction mixture was refluxed for 3 h and then cooled to room temperature. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography to afford the title compound.

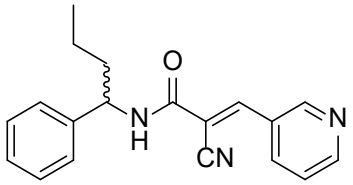
(*E*)-3-(6-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide ((*R*,*S*)-DGS)[5]



(*R*'*S*)-DGS

Prepared according to the general method using 6-bromo-2-pyridinecarboxaldehyde (49 mg, 0.26 mmol). Purification was conducted by column chromatography (30% EtOAc in hexane) to afford **(*R*,*S*)-DGS** as a white solid (59 mg, 64%). ^{1}H NMR (300 MHz, *CDCl*$_3$): 8.18 (s, 1H), 7.68 – 7.55 (m, 3H), 7.39 – 7.26 (m, 5H), 6.78 (d, *J* = 8.0 Hz, 1H), 5.08 (q, *J* = 7.6 Hz, 1H), 1.93 – 1.82 (m, 2H), 1.47 – 1.24 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl*$_3$): 158.75, 151.03, 148.39, 142.60, 141.47, 139.27, 130.78, 128.97, 127.86, 126.65, 125.57, 115.95, 109.64, 54.79, 38.33, 19.59, 13.88. HRMS (ESI) calcd. for $C_{19}H_{18}BrN_3O$ $[M+H]^+$ 384.0706, found 384.0706.

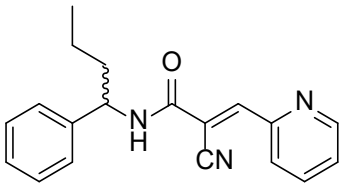
(*E*)-2-Cyano-*N*-(1-phenylbutyl)-3-(pyridin-3-yl)acrylamide (1)



1

Prepared according to the general method using 3-picolinealdehyde (54 mg, 0.51 mmol). Purification was conducted by column chromatography (30% EtOAc in hexane) to afford **1** as a brown solid (48 mg, 34%). ^{1}H NMR (300 MHz, *CDCl*$_3$): 8.90 (d, *J* = 2.3 Hz, 1H), 8.71 – 8.69 (m, 1H), 8.40 – 8.36 (m, 1H), 8.30 (s, 1H), 7.44 – 7.27 (m, 6H), 6.63 (d, *J* = 8.0 Hz, 1H), 5.08 (q, *J* = 7.6 Hz, 1H), 1.97 – 1.78 (m, 2H), 1.46 – 1.27 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl*$_3$): 158.75, 152.94, 152.31, 149.56, 141.38, 135.87, 128.92, 127.96, 127.83, 126.62, 124.02, 116.41, 106.76, 54.76, 38.15, 19.52, 13.81. HRMS (ESI) calcd. for $C_{19}H_{19}N_3O$ $[M+H]^+$ 306.1601, found 306.1600.

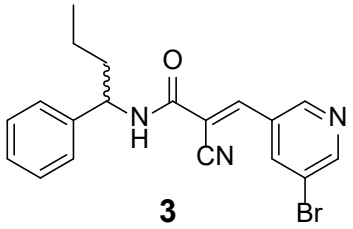
(*E*)-2-Cyano-*N*-(1-phenylbutyl)-3-(pyridin-2-yl)acrylamide (2)



2

Prepared according to the general method using 2-picolinealdehyde (38 mg, 0.36 mmol). Purification was conducted by column chromatography (30% EtOAc in hexane) to afford **2** as a brown solid (41 mg, 42%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 8.82 – 8.79 (m, 1H), 8.27 (s, 1H), 7.82 – 7.76 (m, 1H), 7.60 – 7.57 (m, 1H), 7.41 – 7.26 (m, 6H), 6.80 (d, *J* = 7.6 Hz, 1H), 5.08 (q, *J* = 7.6 Hz, 1H), 1.97 – 1.79 (m, 2H), 1.47 – 1.25 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). – ^{13}C NMR (75 MHz, *CDCl$_3$*): 159.18, 150.65, 150.44, 150.27, 141.59, 137.07, 128.94, 127.80, 127.22, 126.66, 126.10, 116.85, 108.09, 54.71, 38.39, 19.59, 13.89. – HRMS (ESI) calcd. for $C_{19}H_{19}N_3O$ $[M+H]^+$ 306.1601, found 306.1600.

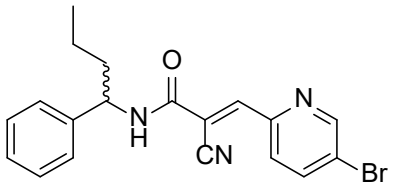
(*E*)-3-(5-Bromopyridin-3-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (3)



3

Prepared according to the general method using 5-bromonicotinaldehyde (71 mg, 0.38 mmol). Purification was conducted by column chromatography (30% EtOAc in hexane) to afford **3** as a yellow solid (48 mg, 36%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 8.85 (d, *J* = 2.0 Hz, 1H), 8.77 (d, *J* = 2.2 Hz, 1H), 8.44 (t, *J* = 2.2 Hz, 1H), 8.24 (s, 1H), 7.39 – 7.29 (m, 5H), 6.59 (d, *J* = 8.0 Hz, 1H), 5.07 (q, *J* = 7.6 Hz, 1H), 1.94 – 1.82 (m, 2H), 1.44 – 1.27 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*): 158.25, 153.99, 149.92, 147.82, 141.23, 138.21, 129.39, 129.02, 127.99, 126.68, 121.40, 115.98, 108.11, 54.92, 38.19, 19.59, 13.87. HRMS (ESI) calcd. for $C_{19}H_{18}BrN_3O$ $[M+H]^+$ 384.0706, found 384.0704.

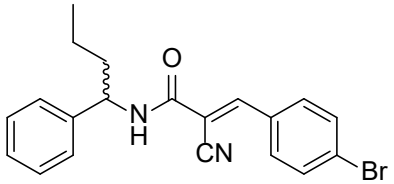
(*E*)-3-(5-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (4)



4

Prepared according to the general method using 5-bromopyridine-2-carbaldehyde (47 mg, 0.25 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **4** as a white solid (45 mg, 51%). ^{1}H NMR (300 MHz, *CDCl*$_3$): 8.85 (d, *J* = 2.3 Hz, 1H), 8.21 (s, 1H), 7.92 (dd, *J* = 8.3, 2.3 Hz, 1H), 7.48 – 7.25 (m, 6H), 6.77 (d, *J* = 8.1 Hz, 1H), 5.07 (q, *J* = 7.6 Hz, 1H), 1.97 – 1.78 (m, 2H), 1.47 – 1.25 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl*$_3$): 158.93, 151.74, 149.16, 148.58, 141.47, 139.69, 128.95, 127.93, 127.83, 126.65, 123.80, 116.52, 108.58, 54.77, 38.33, 19.57, 13.87. HRMS (ESI) calcd. for $C_{19}H_{18}BrN_3O$ $[M+H]^+$ 384.0706, found 384.0711.

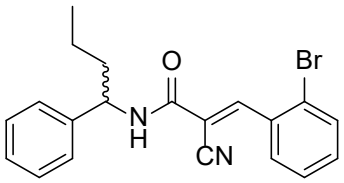
(*E*)-3-(4-Bromophenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (5)



5

Prepared according to the general method using 4-bromobenzaldehyde (47 mg, 0.25 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **5** as a white solid (50 mg, 56%). ^{1}H NMR (300 MHz, *CDCl*$_3$): 8.23 (s, 1H), 7.78 – 7.74 (m, 2H), 7.64 – 7.59 (m, 2H), 7.39 – 7.26 (m, 5H), 6.55 (d, *J* = 8.1 Hz, 1H), 5.07 (q, *J* = 7.6 Hz, 1H), 1.97 – 1.78 (m, 2H), 1.47 – 1.25 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl*$_3$): 159.23, 151.86, 141.53, 132.75, 131.94, 130.77, 128.99, 127.88, 127.73, 126.68, 116.94, 104.77, 54.75, 38.32, 19.59, 13.89. HRMS (ESI) calcd. for $C_{20}H_{19}BrN_2O$ $[M+H]^+$ 383.0754, found 383.0755.

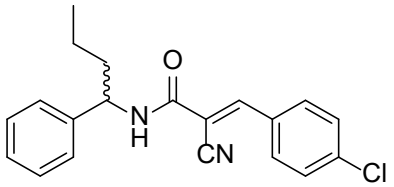
(*E*)-3-(2-Bromophenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (6)



6

Prepared according to the general method using *o*-bromobenzaldehyde (47 mg, 0.25 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **6** as clear oil (62 mg, 70%). ^{1}H NMR (300 MHz, *CDCl₃*): 8.67 (s, 1H), 8.03 (dd, *J* = 7.8, 1.7 Hz, 1H), 7.68 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.46-7.26 (m, 7H), 6.57 (d, *J* = 8.4 Hz, 1H), 5.08 (q, *J* = 7.6 Hz, 1H), 1.99 – 1.79 (m, 2H), 1.45 – 1.23 (m, 2H), 0.96 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl₃*): 158.76, 152.38, 141.48, 133.78, 133.30, 132.37, 129.78, 129.01, 128.06, 127.91, 126.75, 126.56, 116.42, 107.66, 54.84, 38.31, 19.62, 13.89. HRMS (ESI) calcd. for $C_{20}H_{19}BrN_2O$ $[M+H]^+$ 383.0754, found 383.0754.

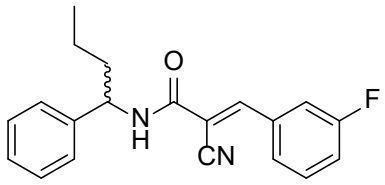
(*E*)-3-(4-Chlorophenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (7)



7

Prepared according to the general method using p-chlorobenzaldehyde (33 mg, 0.23 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **7** as a white solid (42 mg, 54%). ^{1}H NMR (300 MHz, *CDCl₃*): 8.26 (s, 1H), 7.78 – 7.82 (m, 2H), 7.48 – 7.27 (m, 7H), 6.56 (d, *J* = 8.1 Hz, 1H), 5.07 (q, *J* = 7.6 Hz, 1H), 1.96 – 1.78 (m, 2H), 1.47 – 1.23 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl₃*): 159.24, 151.74, 141.54, 139.10, 131.87, 130.37, 129.74, 128.97, 127.85, 126.66, 116.93, 104.61, 54.73, 38.32, 19.59, 13.99. HRMS (ESI) calcd. for $C_{20}H_{19}ClN_2O$ $[M+H]^+$ 339.1259, found 339.1257.

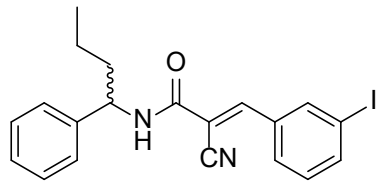
(*E*)-2-Cyano-3-(3-fluorophenyl)-*N*-(1-phenylbutyl)acrylamide (8)



8

Prepared according to the general method using 3-fluorobenzaldehyde (27 mg, 0.25 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **8** as a white solid (50 mg, 67%). ^{1}H NMR (400 MHz, *CDCl$_3$*): 8.26 (s, 1H), 7.67 – 7.60 (m, 2H), 7.48 – 7.20 (m, 7H), 6.58 (d, *J* = 8.2 Hz, 1H), 5.07 (q, *J* = 7.6 Hz, 1H), 1.96 – 1.81 (m, 2H), 1.45 – 1.27 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, *CDCl$_3$*): 162.85 (d, $^{1}J_{C\text{-}F}$ = 330 Hz), 159.18, 151.74, 141.50, 133.91, 133.81, 131.02, 130.91, 128.96, 127.85, 126.67, 119.80 (d, $^{2}J_{C\text{-}F}$ = 28 Hz), 116.83 (d, $^{2}J_{C\text{-}F}$ = 30 Hz), 105.71, 54.76, 38.28, 19.58, 13.87. HRMS (ESI) calcd. for $C_{20}H_{19}FN_2O$ $[M+H]^+$ 323.1554, found 323.1554.

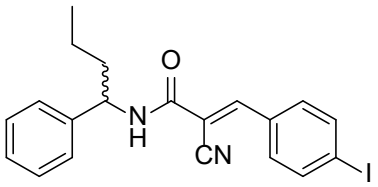
(*E*)-2-Cyano-3-(3-iodophenyl)-*N*-(1-phenylbutyl)acrylamide (9)



9

Prepared according to the general method using 3-iodobenzaldehyde (54 mg, 0.23 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **9** as clear oil (34 mg, 34%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 8.11 (s, 1H), 8.07 (t, *J* = 1.8 Hz, 1H), 7.85-7.75 (m, 2H), 7.32-7.11 (m, 6H), 6.50 (d, *J* = 8.1 Hz, 1H), 5.00 (q, *J* = 7.6 Hz, 1H), 1.90 – 1.71 (m, 2H), 1.39 – 1.18 (m, 2H), 0.88 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*): 158.99, 151.36, 141.47, 139.43, 133.93, 130.85, 129.14, 128.97, 127.87, 126.68, 116.62, 116.60, 105.65, 94.79, 54.75, 38.29, 19.58, 13.87. HRMS (ESI) calcd. for $C_{20}H_{19}IN_2O$ $[M+H]^+$ 431.0615, found 431.0613.

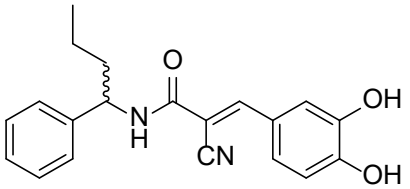
(*E*)-2-Cyano-3-(4-iodophenyl)-*N*-(1-phenylbutyl)acrylamide (10)



10

Prepared according to the general method using 4-iodobenzaldehyde (54 mg, 0.23 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **10** as a white solid (75 mg, 75%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 8.14 (s, 1H), 7.75 (d, *J* = 8.5 Hz, 1H), 7.53 (d, *J* = 8.5 Hz, 1H), 7.31-7.18 (m, 5H), 6.49 (d, *J* = 8.1 Hz, 1H), 4.99 (q, *J* = 7.6 Hz, 1H), 1.89 – 1.71 (m, 2H), 1.39 – 1.18 (m, 2H), 0.87 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*): 159.21, 152.04, 141.51, 138.71, 131.77, 131.26, 128.96, 127.85, 126.66, 116.91, 104.84, 100.28, 54.73, 38.30, 19.58, 13.88. HRMS (ESI) calcd. for $C_{20}H_{19}IN_2O$ $[M+H]^+$ 431.0615, found 431.0613.

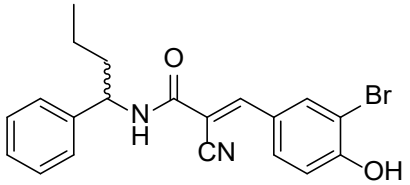
(*E*)-2-Cyano-3-(3,4-dihydroxyphenyl)-*N*-(1-phenylbutyl)acrylamide (11)



11

Prepared according to the general method using 3,4-dihydroxybenzaldehyde (56 mg, 0.41 mmol). Purification was conducted by column chromatography (50% EtOAc in hexane) to afford **11** as a yellow solid (85 mg, 68%). ^{1}H NMR (400 MHz, *DMSO-d$_6$*): 9.83 (brs, 2H), 8.61 (d, *J* = 8.2 Hz, 1H), 7.91 (s, 1H), 7.54 (s, 1H), 7.38 – 7.20 (m, 6H), 6.88 (d, *J* = 8.2 Hz, 1H), 4.88 (q, *J* = 6.0 Hz, 1H), 1.90 – 1.65 (m, 2H), 1.41 – 1.17 (m, 2H), 0.89 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, *DMSO-d$_6$*): 161.56, 150.59, 150.27, 145.67, 143.53, 128.23, 126.76, 126.56, 125.06, 123.34, 117.14, 116.10, 115.91, 101.33, 53.48, 37.71, 19.37, 13.59. HRMS (ESI) calcd. for $C_{20}H_{20}N_2O_3$ $[M+H]^+$ 337.1547, found 337.1546.

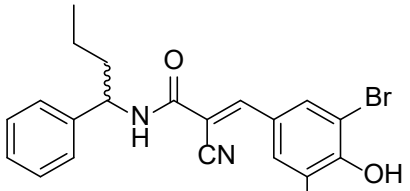
(*E*)-3-(3-Bromo-4-hydroxyphenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (12)



12

Prepared according to the general method using 3-bromo-4-hydroxybenzaldehyde (82 mg, 0.41 mmol). Purification was conducted by column chromatography (50% EtOAc in hexane) to afford **12** as a pale yellow solid (100 mg, 67%). ^{1}H NMR (400 MHz, *DMSO-d*$_6$): 11.42 (s, 1H), 8.69 (d, *J* = 8.2 Hz, 1H), 8.17 (q, *J* = 2.2 Hz, 1H), 8.00 (s, 1H), 7.86 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.38 – 7.21 (m, 5H), 7.10 (d, *J* = 8.6 Hz, 1H), 4.88 (q, *J* = 7.8 Hz, 1H), 1.89 – 1.65 (m, 2H), 1.41 – 1.20 (m, 2H), 0.89 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, *DMSO-d*$_6$): 161.08, 157.91, 148.55, 143.37, 135.25, 131.31, 128.22, 126.78, 126.55, 126.54, 124.46, 116.71, 116.69, 109.96, 103.45, 53.53, 37.70, 19.33, 13.58. HRMS (ESI) calcd. for $C_{20}H_{19}BrN_2O_2$ $[M+H]^+$ 399.0703, found 399.0703.

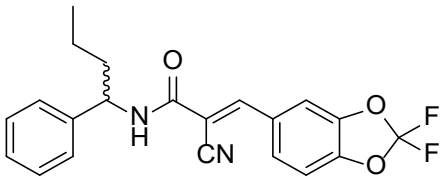
(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (13)



13

Prepared according to the general method using 5-bromovanillin (59 mg, 0.25 mmol). Purification was conducted by column chromatography (40% EtOAc in hexane) to afford **13** as a pale yellow solid (62 mg, 63%). ^{1}H NMR (300 MHz, *CDCl*$_3$): 8.13 (s, 1H), 7.61 (d, *J* = 2.0 Hz, 1H), 7.56 (d, *J* = 2.0 Hz, 1H), 7.39 – 7.25 (m, 5H), 6.51 (brs, 1H), 6.49 (s, 1H), 5.07 (q, *J* = 7.6 Hz, 1H), 3.96 (s, 3H), 1.92 – 1.80 (m, 2H), 1.45 – 1.25 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl*$_3$): 159.63, 151.76, 147.49, 147.39, 141.64, 130.18, 128.94, 127.81, 126.67, 125.17, 117.48, 110.16, 108.77, 102.05, 56.71, 54.62, 28.36, 19.60, 13.90. HRMS (ESI) calcd. for $C_{21}H_{21}BrN_2O_3$ $[M+H]^+$ 429.0808, found 429.0807.

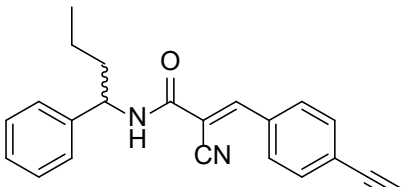
(*E*)-2-Cyano-3-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)-*N*-(1-phenylbutyl)acrylamide (14)



14

Prepared according to the general method using 2,2-difluoro-5-formylbenzodioxole (43 mg, 0.23 mmol). Purification was conducted by column chromatography (10% EtOAc in hexane) to afford **14** as clear oil (40 mg, 40%). ^{1}H NMR (300 MHz, *CDCl₃*): 8.16 (s, 1H), 7.70 (d, *J* = 1.8 Hz, 1H), 7.52-7.48 (m, 1H), 7.32-7.18 (m, 5H), 7.09 (d, *J* = 8.4 Hz, 1H), 6.48 (d, *J* = 8.1 Hz, 1H), 4.99 (q, *J* = 7.6 Hz, 1H), 1.90 – 1.71 (m, 2H), 1.39 – 1.18 (m, 2H), 0.88 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl₃*): 159.15, 151.50, 146.61, 144.48, 141.51, 131.75 (t, $^{1}J_{C\text{-}F}$ = 257 Hz), 129.01, 128.94, 128.22, 127.84, 126.65, 116.83, 110.24, 110.17, 104.15, 54.76, 38.27, 19.57, 13.84. HRMS (ESI) calcd. for $C_{21}H_{18}F_2N_2O_3$ $[M+H]^+$ 385.1358, found 385.1356.

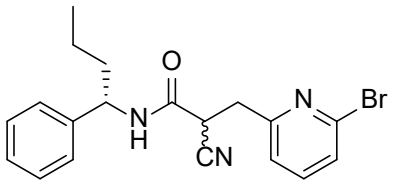
(*E*)-2-Cyano-3-(4-ethynylphenyl)-*N*-(1-phenylbutyl)acrylamide (15)



15

Prepared according to the general method using 4-ethynylbenzaldehyde (33 mg, 0.25 mmol). Purification was conducted by column chromatography (20% EtOAc in hexane) to afford **15** as a white solid (49 mg, 65%). ^{1}H NMR (300 MHz, *CDCl₃*): 8.27 (s, 1H), 7.88-7.84 (m, 2H), 7.59-7.55 (m, 2H), 7.39-7.25 (m, 5H), 6.57 (d, *J* = 8.1 Hz, 1H), 5.08 (q, *J* = 7.6 Hz, 1H), 3.27 (s, 1H), 1.98 – 1.79 (m, 2H), 1.50 – 1.24 (m, 2H), 0.96 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl₃*): 159.24, 151.96, 141.54, 132.87, 132.00, 130.47, 128.94, 127.82, 126.65, 126.62, 116.94, 104.89, 82.83, 81.02, 54.71, 38.29, 19.57, 13.87. HRMS (ESI) calcd. for $C_{22}H_{20}N_2O$ $[M+H]^+$ 329.1648, found 329.1648.

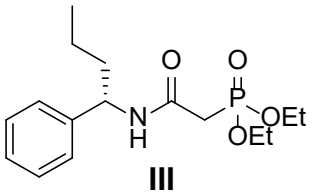
3-(6-Bromopyridin-2-yl)-2-cyano-*N*-((*S*)-1-phenylbutyl)propanamide (16)



16

To a solution of (*S,E*)-3-(6-bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (**DGS**, 83 mg, 0.22 mmol) in THF (2.0 mL) was added $NaBH_4$ (5.0 mg, 0.13 mmol) at 0 °C. The mixture was warmed to room temperature after 24h and quenched with water. The aqueous phase was extracted with EtOAc three times and the combined organic layers were dried over Na_2SO_4. The residue was concentrated under reduced pressure and purified by column chromatography (20% EtOAc in hexane) to afford the diastereomers of **16** as a yellow solid (48 mg, 58%). ^{1}H NMR (500 MHz, *CDCl$_3$*): 7.51 – 7.21 (m, 12H), 7.18 – 7.14 (m, 4H), 6.67 (d, *J* = 8.2 Hz, 1H), 6.58 (d, *J* = 8.2 Hz, 1H), 4.88 (q, *J* = 7.7 Hz, 2H), 4.12 (t, *J* = 6.8 Hz, 1H), 4.07 (t, *J* = 6.8 Hz, 1H), 3.51 – 3.41 (m, 2H), 3.35 – 3.27 (m, 2H), 1.85 – 1.70 (m, 4H), 1.34 – 1.16 (m, 4H), 0.91 (t, *J* = 7.3 Hz, 3H), 0.86 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (125 MHz, *CDCl$_3$*): 163.31, 157.11, 157.03, 141.74, 141.73, 141.51, 141.49, 139.44, 139.37, 127.76, 127.67, 127.07, 127.04, 122.92, 122.84, 117.75, 117.66, 54.52, 54.47, 38.31, 38.27, 37.20, 37.09, 36.75, 36.65, 19.58, 19.51, 13.91, 13.87. HRMS (ESI) calcd. for $C_{19}H_{20}BrN_3O$ $[M+H]^+$ 386.0863, found 386.0865.

Diethyl (*S*)-(2-oxo-2-((1-phenylbutyl)amino)ethyl)phosphonate (III)

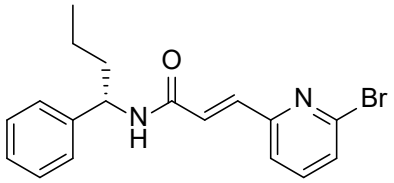


III

A mixture of 4-dimethylaminopyridine (126 mg, 1.03 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (198 mg, 1.03 mmol) was added to a stirred solution of diethylphosphonoacetic acid (101 mg, 0.516 mmol) and (*S*)-1-phenylbutan-1-amine (77 mg, 0.51 mmol) in DMF. The resulting mixture was stirred under argon for 3 h at room temperature and then poured into EtOAc. The solution was washed twice with 1N HCl and once with brine, dried over Na_2SO_4 and concentrated. The crude product was purified by column chromatography (70% EtOAc in hexane) to yield compound **III** as a yellow oil (124 mg, 73%).

^{1}H NMR (300 MHz, *CDCl$_3$*): 7.31 – 7.15 (m, 5H), 4.95 (q, *J* = 7.9 Hz, 2H), 4.16 – 3.97 (m, 4H), 2.96 – 2.76 (m, 2H), 1.85 – 1.67 (m, 2H), 1.42 – 1.18 (m, 8H), 0.91 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*): 163.10, 142.46, 128.66, 127.34, 126.65, 63.06 (t, $^{1}J_{C\text{-}P}$ = 70 Hz), 53.87, 38.66, 36.06, 34.33, 19.50, 16.45 (d, $^{2}J_{C\text{-}F}$ = 6.1 Hz), 16.33 (d, $^{2}J_{C\text{-}F}$ = 6.1 Hz), 13.91. HRMS (ESI) calcd. for $C_{18}H_{19}BrN_2O$ $[M+H]^+$ 328.1672, found 328.1676.

(*S,E*)-3-(6-Bromopyridin-2-yl)-*N*-(1-phenylbutyl)acrylamide (17)



17

To a solution of sodium hydride (60% wt. in mineral oil, 13 mg, 0.32 mmol) in dry THF (0.5 mL) was added a solution of diethyl (*S*)-(2-oxo-2-((1-phenylbutyl)amino)ethyl)phosphonate **III** (34 mg, 0.10 mmol) in THF (1.0 mL) at 0 °C. The mixture was warmed to room temperature and stirred for 30 min. Then a solution of 6-bromopicolinaldehyde (19 mg, 0.10 mmol) in dry THF (1 mL) was added dropwise and stirred overnight. The reaction was quenched with water, and the aqueous phase was extracted with EtOAc three times. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (40% EtOAc in hexane) to afford **17** as a colorless solid (21 mg, 56%). ^{1}H NMR (300 MHz, *CDCl$_3$*): 7.47 – 7.14 (m, 9H), 6.96 (d, *J* = 15.0 Hz, 1H), 6.01 (d, *J* = 8.4 Hz, 1H), 5.02 (q, *J* = 7.7 Hz, 2H), 4.12 (t, *J* = 6.8 Hz, 1H), 4.07 (t, *J* = 6.8 Hz, 1H), 1.79 – 1.68 (m, 2H), 1.37 – 1.16 (m, 2H), 0.85 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, *CDCl$_3$*): 164.41, 154.48, 142.53, 142.26, 139.23, 137.85, 128.80, 128.27, 127.51, 126.67, 126.59, 123.54, 53.66, 38.54, 19.60, 13.96. HRMS (ESI) calcd. for $C_{18}H_{19}BrN_2O$ $[M+H]^+$ 359.0754, found 359.0759.

5. Biochemical Procedures

5.1 *S. aureus* strains and media

Staphylococcus aureus strains were obtained from Institute Pasteur, France (NCTC8325 and MU50) or the group of Professor Markus Gerhard at the Institute of Medical Microbiology, Immunology and Hygiene (Technische Universität München) (VA402525, VA409044, VA412350, VA402923, VA418879, VA417350, IS050611, BK097296, IS050678, BK095395). All *S. aureus* strains were cultured in B-medium (LB-Broth 20.0 g/L (Yeast extracts 5.0 g/L, Tryptic peptone 10.0 g/L, NaCl 5.0 g/L), K_2HPO_4 1.0 g/L). The resistance patterns of the clinical isolates have been reported before.[6]

5.2 Overnight cultures of bacteria and cryostocks

5 mL of B-medium were inoculated with 5 µL of the desired bacterial cryostock (1:1,000) in a plastic culture tube. The culture was incubated overnight (routinely 16 h) by shaking at 200 rpm at 37 °C. All overnight cultures were prepared freshly to avoid genetic variation. A sterile control was added each time (medium without bacteria).

5 mL of an overnight culture of the desired bacteria were harvested by centrifugation (6,000 g, 10 m, 4 °C) and the supernatant was removed. The living bacteria pellet was resuspended in 750 µL of fresh B-medium. Sterile glycerol was added to a final concentration of 50%. The stock was mixed and stored at -80 °C in 20 µL aliquots prior to use. After inoculating fresh media with the aliquot, the leftover of the cryostock was discarded.

5.3 Minimal inhibitory concentration (MIC) assay

The overnight culture was diluted to $OD_{600} = 0.001$ in fresh B-media and 100 µL aliquots were added to 96 well plates which contain various concentrations of compounds (1 µL in DMSO) or DMSO (1 µL) as a negative control. Sterile controls were performed on each plate with 100 µL B-medium and 1 µL DMSO. After 20 h incubation at 200 rpm at 37 °C, the optical density was measured at 600 nm with a plate reader (infinite M200Pro plate reader). The MIC was determined as the lowest concentration, for which no bacterial growth was detected.

5.4 Analytical gel-based ABPP

An overnight culture of *S. aureus* NCTC 8325 was grown in 5 mL B medium in a plastic culture tube. After 1:100 dilution of the culture with fresh B-medium, the cultures were grown until stationary phase. Cultures were collected in a 50 mL falcon tube and centrifuged at 6,000 g for 10 min at 4 °C. The supernatant was disposed and the pellet was resuspended in PBS to reach OD_{600} = 40. 200 µL of this suspension and 2 µL of probe in DMSO (or DMSO as a control) were mixed and incubated for 1 h at 200 rpm at 37 °C. The bacterial suspension was centrifuged at 6,000 g for 10 min at 4 °C. The supernatant was removed and the pellets were stored at –80 °C. Pellets were resuspended in 200 µL PBS (4 °C) and the suspension was lysed by sonication with five times 20 sec pulses at 80% max. power on ice. The suspension was incubated two times for 0.5 h at 200 rpm at 37 °C after addition of 2 µL of lysostaphin (2 mg/mL) and then 10 µL of 10% (w/v) SDS in PBS solution in that order. Click chemistry was carried out with 20 µL click reagent mix (4 µL RhN_3 (5 mM Rhodamine azide in DMSO), 4 µL TCEP (50 mM tris(2-carboxyethyl)phosphine in ddH_2O), 12 µL TBTA ligand (1.667 mM Tris(benzyltriazolylmethyl)amine in 80% tBuOH and 20% DMSO)). The lysates were mixed by vortexing and 4 µL $CuSO_4$ solution (50 mM in ddH_2O) were added to start the click reaction. The lysates were mixed by vortexing again and incubated for 1 h at RT in the dark. Then 200 µL 2x Laemmli Sample Buffer were added, vortexed and analysed via SDS-PAGE (12.5% agarose gel (PEQLAB Biotechnilogie GmbH, Erlangen, PerfectBule Dual Gel System, gels were prepared according to the manual), 2.5 h, 150 V, 8 µL fluorescent protein standard and fluorescence imaging (GE Healthcare, ImageQuant LAS-4000)).

5.5 Conventional activity-based protein profiling (ABPP)

Sample preparation

An overnight culture of *S. aureus* was grown in 5 mL B medium in a plastic culture tube. After 1:100 dilution of the culture with fresh B-medium, the cultures were grown until stationary phase. Cultures were collected in a 50 mL falcon tube and centrifuged at 6,000 g for 10 min at 4 °C. The supernatant was disposed and the pellet was resuspended in PBS to reach OD_{600} = 40. 500 µL of this suspension and 5 µL of probe in DMSO (or DMSO as a control) were mixed and incubated for 1 h at 200 rpm at 37 °C. The bacterial suspension was centrifuged at 6,000 g for 10 min at 4 °C. The supernatant was removed and the pellets were stored at –80 °C. Pellets were resuspended in 500 µL PBS (4 °C) and the suspension was lysed by sonication with five

times 20 sec pulses at 80% max. power on ice. The suspension was incubated two times for 0.5 h at 200 rpm at 37 °C after addition of 5 µL of lysostaphin (2 mg/mL) and then 25 µL of 10% (w/v) SDS in PBS solution in that order. Protein concentration was determined using a bicinchoninic acid (BCA) assay and the concentration was adjusted to 1 mg/mL with PBS. In a 15 mL falcon tube, 500 µL of solution were treated with 43 µL click reagent mix (3 µL Biotin-PEG3-N_3 (Jena Bioscience, CLK-AZ104P4-100; 10 mM in DMSO), 10 µL TCEP (50 mM in ddH_2O), 30 µL TBTA ligand (1.667 mM in 80% tBuOH and 20% DMSO)) resulting in final concentrations of 233 µM Biotin-PEG3-N_3, 581 µM TCEP and 58.2 µM TBTA Ligand. The lysates were mixed by vortexing and 10 µL $CuSO_4$ solution (50 mM in ddH_2O) were added to start the click reaction. The lysates were mixed by vortexing again and incubated for 1 h at RT in the dark. Subsequently 4 mL of cold acetone (–80° C, MS grade) were added and proteins were precipitated overnight at –80 °C. The precipitated proteins were thawed on ice, pelletized (10,000 g, 15 min, 4 °C) and the supernatant was disposed. Proteins were washed two times with 1 mL cold methanol (–80 °C, MS grade). Resuspension was achieved by sonication (10 sec at 10% intensity) and proteins were pelletized *via* centrifugation (10,000 g, 10 min, 4 °C). After the washing steps the supernatant was disposed and the pellet resuspended in 500 µL 0.4% SDS in PBS at RT by sonication (10 sec at 10% intensity). 50 µL avidin-agarose beads (Sigma-Aldrich) were prepared by washing three times with 1 mL 0.4% SDS in PBS. All centrifugation steps were conducted at 400 g for 3 min at RT. 500 µL protein solution was added to the washed avidin-agarose beads and incubated under continuous inverting (1 h, RT). Beads were washed three times with 1 mL 0.4% SDS in PBS, two times with 6 M urea in ddH_2O and three times with 1 mL PBS.

Digestion

The beads were resuspended in 200 µL denaturation buffer (7 M urea, 2 M thiourea in 20 mM pH 7.5 HEPES buffer). Dithiothreitol (DTT, 500 mM, 0.4 µL) was added, the tubes were mixed by vortexing shortly and incubated in a thermoshaker (600 rpm, 60 min, RT). Then 2-iodoacetamide (IAA, 500 mM, 4 µL) was added, the tubes were mixed by vortexing shortly and incubated in a thermoshaker (600 rpm, 30 min, RT, in the dark). Remaining IAA was quenched by the addition of dithiothreitol (DTT, 500 mM, 4 µL). The tubes were shortly mixed by vortexing and incubated in a thermoshaker (600 rpm, 30 min, RT). LysC (0.5 µg/µL) was thawed on ice and 1 µL was added to each microcentrifuge tube, the tubes were shortly mixed by vortexing and incubated in a thermoshaker (600 rpm, 2 h, RT, in the dark). TEAB solution (600 µL, 50 mM in water) and then trypsin (1.5 µL, 0.5 µg/µL in 50 mM acetic acid) were

added to each tube with a short vortexing step after each addition. The microcentrifuge tubes were incubated in a thermoshaker (600 rpm, 15 h, 37 °C). The digest was stopped by adding 10 µL formic acid (FA) and vortexing followed by centrifugation (13,000 g, 3 min, RT).

Desalting

50 mg SepPak C18 columns (Waters) were equilibrated by gravity flow two times with 1 mL acetonitrile and three times with 1 mL aqueous 0.1% trifluoroacetic acid (TFA) solution. Subsequently the samples were loaded by gravity flow, washed three times with 1 mL aqueous 0.1% TFA solution and once with 0.5 mL aqueous 0.5% FA solution. Elution of proteins into new 2.0 mL Protein LoBind Eppendorf tubes was performed by two times addition of 250 µL elution buffer (80% ACN, 0.5% FA) by gravity flow followed by 250 µL elution buffer by vacuum flow until all liquid was eluted from the column. The eluates were lyophilized.

Filtering and MS measurement

Before MS measurement the samples were dissolved in 30 µL 1% FA by pipetting up and down, vortexing and sonication for 15 min (brief centrifugation after each step). 0.22 µm centrifugal filter units (VWR) were equilibrated with 300 µL 1% FA (13,000 g, 2 min, RT) and samples were filtered through the equilibrated filters (centrifugation: 13,000 g, 1 min, RT).

Samples were analyzed with an UltiMate 3000 nano HPLC system (Dionex) using Acclaim C18 PepMap100 75 µm ID x 2 cm trap and Acclaim PepMap RSLC C18 (75 µm ID x 50 cm) separation columns in an EASY-spray setting coupled to a Q Exactive Plus mass spectrometer (ThermoFisher). 5 µL peptide samples were loaded on the trap and washed with 0.1% TFA, then transferred to the analytical column (buffer A: H_2O with 0.1% FA, buffer B: MeCN with 0.1% FA, flow 0.3 µL/min, gradient: to 5% buffer B in 7 min, from 5% to 22% buffer B in 105 min, then to 32% buffer B in 10 min, to 90% buffer B in 10 min and hold at 90% buffer B for 10 min, then to 5% buffer B in 0.1 min and hold 5% buffer B for 9.9 min) and ionized at spray voltage of 2.0 kV and a capillary temperature of 275 °C. The Q Exactive Plus mass spectrometer was operated in a TOP12 data dependent mode with full scan acquisition in the orbitrap at a resolution of R = 140,000 and an AGC target of 3e6 in a scan range of 300 - 1500 m/z with a maximum injection time of 80 ms. Monoisotopic precursor selection as well as dynamic exclusion (dynamic exclusion duration: 60 s) was enabled. Precursors with charge states >1 and intensities greater than 1e4 were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z. Precursors were analyzed in a scan range of 200 – 2000 m/z to an AGC target of 1e5 and a maximum injection time of 100 ms.

Peptide fragments were generated by higher-energy collisional dissociation (HCD) with a normalized collision energy of 27% and detected in the orbitrap.

Peptide and protein identifications were performed using MaxQuant 1.6.0.16 software with Andromeda as search engine using the following parameters: Carbamidomethylation of cysteines as fixed and oxidation of methionine as dynamic modification, trypsin/P as the proteolytic enzyme, 4.5 ppm for precursor mass tolerance (main search ppm) and 0.5 Da for fragment mass tolerance (ITMS MS/MS tolerance). Searches were performed against the Uniprot database for S. aureus NCTC8325 (taxon identifier: 93061, downloaded on 12.09.2018). Quantification was performed using label-free quantification (LFQ). The match between runs (default settings) option was used. Identification was done with at least 2 unique peptides and quantification only with unique peptides.

Statistical analysis was performed with Perseus 1.6.2.2. LFQ ratios were log2(x) transformed. $-\log_{10}$(p-values) were obtained by a two sample t-test over four biological replicates. Putative contaminants, reverse peptides and peptides only identified by site were deleted. Data was filtered for three valid values in at least one group and a missing value imputation was performed over the total matrix.

5.6 Competition experiment

The same procedure as described for the enrichment studies was followed with the following exception: The bacterial suspension was pre-treated with **DGS** in DMSO (or DMSO as a control) for 45 min at 200 rpm at 37 °C, and then treated with 12.5 µM **15** in DMSO for 45 min at 200 rpm at 37 °C.

5.7 Competitive isoDTB-ABPP

Sample preparation

Overnight cultures of *S. aureus* NCTC8325 were inoculated with 5 µL of a corresponding glycerol stock into 5 mL of B- medium and grown overnight at 37 °C with shaking at 200 rpm. For preparation of lysates, the indicated medium was inoculated 1:100 with an overnight culture of the respective bacteria and incubated at 37 °C with shaking at 200 rpm until 1 h after it reached the stationary phase. The cells were harvested by centrifuging at 8,000 xg at 4 °C for

10 min, and the pellets were washed two times with PBS prior to the immediate use or storage at −80 °C. PBS was added to the bacterial pellets and the pellets were resuspended and transferred into 7 mL tubes containing 0.1 mm ceramic beads. Cells were lysed in a Precellys 24 bead mill using three 30 s cycles at 6,500 rpm while cooling with an airflow that was pre-cooled with liquid nitrogen. The suspension was transferred into an Eppendorf tube and centrifuged at 20,000 xg at 4 °C for 30 min. The supernatant of several samples was pooled and filtered through a 0.45 µm filter. Protein concentration of the lysate was determined using a bicinchoninic acid (BCA) assay and the concentration was adjusted to 1 mg/mL with PBS.

1.00 mL freshly prepared lysate were incubated with 10 µL 100x of **DGS** in DMSO (e.g. 10 µL 10 mM stock for 100 µM final concentration or 10 µL 2 mM stock for 20 µM final concentration) at room temperature for 1 h. Another 1.00 mL sample of lysate was incubated with 10 µL of DMSO. After this incubation, 20 µL of 50 mM IA-alkyne in DMSO were separately added to the lysate with and without competitor and incubated at room temperature for 1 h. The samples were clicked to the heavy (DMSO-treated) and light (compound-treated) isoDTB tags by adding 120 µL of a solution consisting of 60 µL 0.9 mg/mL TBTA ligand in 4:1 tBuOH/DMSO, 20 µL 12.5 mg/mL $CuSO_4$ in water, 20 µL 13 mg/ml TCEP in water and 20 µL 5 mM of the respective isoDTB tag in DMSO. After incubation of the samples at room temperature for 1 h, the light- and heavy-labeled samples were combined into 8 mL of cold acetone in order to precipitate all proteins. Precipitates were stored at −20 °C overnight.

MS Sample Preparation

The protein precipitates were centrifuged at 3,500 rpm at 25 °C for 10 min. The supernatant was removed, and precipitates resuspended in 1 mL cold methanol by sonification. After centrifugation at 20,000 xg at 4 °C for 10 min, the supernatant was removed. This wash step with methanol was repeated one more time. The pellets were dissolved in 300 µL 8 M urea in 0.1 M triethylammonium bicarbonate (TEAB) by sonification. 900 µL 0.1 M TEAB were added to obtain a concentration of 2 M urea. This solution was added to 1.2 mL of washed high capacity streptavidin agarose beads (50 µL initial slurry, Fisher Scientific, 10733315) in 0.2% nonyl phenoxypolyethoxylethanol (NP40). The samples were rotated at room temperature for 1 h in order to assure binding to the beads.

The beads were centrifuged (1 min, 1,000 xg) and the supernatant was removed. The beads were resuspended in 600 µL 0.1% NP40 in PBS and transferred to a centrifuge column (Fisher Scientific, 11894131). Beads were washed two times with 0.1% NP40 in PBS, three times with PBS and three times with ddH_2O. The beads were resuspended in 600 µL 8 M urea in 0.1 M

TEAB, transferred to a Protein LoBind tube and centrifuged (1 min, 1,000 xg). The supernatant was removed, and the beads were resuspended in 300 µL 8 M urea in 0.1 M TEAB. 15 µL of dithiothreitol (DTT; 31 mg/mL in water) were added and the beads incubated at 37 °C with shaking at 200 rpm for 45 min. Free thiol groups were modified by adding 15 µL of iodoacetamide (74 mg/mL in water) and incubation at 25 °C with shaking at 200 rpm for 30 min. Remaining iodoacetamide was quenched by adding 15 µL DTT (31 mg/mL in water) and incubation at 25 °C with shaking at 200 rpm for another 30 min.

For trypsin digestion, 900 µL TEAB were added to the samples after alkylation to obtain a urea concentration of 2 M. Samples were centrifuged (1 min, 1,000 xg) and the supernatant removed. The beads were resuspended in 200 µL 2 M urea in 0.1 M TEAB. 4 µL 0.5 mg/mL trypsin (Promega, V5113) were added and samples incubated at 37 °C with shaking at 200 rpm overnight. After the digestion, samples were diluted by adding 400 µL of 0.1% NP40 in PBS and transferred to a centrifuge column (Fisher Scientific, 11894131). Beads were washed three times with 0.1% NP40 in PBS, three times with PBS (MS grade) and three times with water (MS grade). Peptides were eluted by adding 200 µL 0.1% formic acid in 50% acetonitrile in water to the column. 100 µL of the elution buffer were added two times more to the column and peptides eluted into a Protein LoBind tube by centrifuging at 3,000 xg for 3 min. The solvent was removed in a vacuum concentrator by rotating at 30 °C for approximately 5 h. Samples were dissolved in 30 µL 1% formic acid in water by sonification for 5 min. Samples were filtered (Merck, UVC30GVNB) washed with the same solvent and transferred into MS sample vials. Samples were stored at −20 °C until measurement.

Sample Analysis by LC-MS/MS

10 µL of the samples were analyzed using a Q Exactive Plus mass spectrometer (ThermoFisher) coupled to an Ultimate 3000 nano HPLC system (Dionex). Samples were loaded on an Acclaim C18 PepMap100 trap column (75 µm ID x 2 cm) and washed with 0.1% TFA. The subsequent separation was carried out on an Acclaim C18PepMapRSLC column (75 µm ID x 50 cm) with a flow of 300 µL/min and buffer A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. Analysis started with washing in 5% B for 7 min followed a gradient from 5% to 40% buffer B in 105 min, an increase to 60% B in 10 min and another increase to 90% B in 10 min. 90% B was held for 10 min, then decreased to 5% in 0.1 min and held at 5% for another 9.9 min. The Q Exactive Plus mass spectrometer was run in a TOP10 data-dependent mode. In the orbitrap, full MS scans were collected in a scan range of 300-1500 m/z at a resolution of 70,000 and an AGC target of 3e6 with 80 ms maximum injection time. The most intense peaks

were selected for MS2 measurement with a minimum AGC target of 1e3 and isotope exclusion and dynamic exclusion (exclusion duration: 60 s) enabled. Peaks with unassigned charge or a charge of +1 were excluded. MS2 spectra were collected at a resolution of 17,500 aiming at an AGC target of 1e5 with a maximum injection time of 100 ms. Isolation was conducted in the quadrupole using a window of 1.6 m/z. Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 27%) and finally detected in the orbitrap.

Data evaluation using MaxQuant

MS raw data were analyzed using MaxQuant software (version 1.6.1.0).[7] Standard settings were used with the following changes and additions: A modified FASTA database, based on the UniProt database for *S. aureus* NCTC8325 (taxon identifier: 93061, downloaded on 12.09.2018), with individual substitutions of cysteines with the placeholder "U" were used as described previously.[8] Labels were set on the placeholder amino acid "U" for the light isoDTB tag as light label ($C_{28}H_{46}N_{10}O_6S_1Se_{-1}$) and the heavy isoDTB tag as heavy label ($C_{24}{}^{13}C_4H_{46}N_8{}^{15}N_2O_6S_1Se_{-1}$). A multiplicity of 2 was set and a maximum number of labeled amino acids of 1. The digestion enzyme was set to Trypsin/P with maximum number of missed cleavages of 2. No variable modifications were included. The "Re-quantify" option was enabled. Carbamidomethyl (C_2H_3NO) was used as fixed modification on cysteine. Contaminants were included. Peptides were searched with a minimum peptide length of 7 and a maximum peptide mass of 4,600 Da. "Second peptides" and "Dependent peptides" were disabled and the option "Match between run" was enabled with a Match time window of 0.7 min and an alignment window of 20 min. An FDR of 0.01 was used for Protein FDR, PSM FDR and XPSM FDR.

Data Processing

The "peptides.txt" file of the MaxQuant analysis was used for further analysis. All peptide sequences without a modified cysteine (placeholder "U") and all reverse sequences were deleted. Only the columns "Sequence", "Leading Razor Protein", "Start Position" and the columns for "Ratio H/L" for all experiments were kept. The "Leading Razor Protein" was renamed to the Uniprot Code without the indicator for the number of the cysteine. All individual ratios were filtered out, if they were "NaN", and all other values were transformed into the log2-scale. For each peptide in each condition, the data was filtered out, if there were not at least two data points for individual experiments or if the standard deviation between the experiments exceeded a value of 1.41. For each peptide, an identifier was generated in the form "Uniprot

Code"_"C""residue number of the modified cysteine". The data for the same experiment for all peptides with the same identifier, and therefore the same modified cysteine, were combined. Here, the median of the data was used. The data was filtered out if the standard deviation exceeded a value of 1.41. Each modified cysteine was kept in the dataset once with the shortest peptide sequence as the reported sequence. For each modified cysteine, all values for the same condition were combined, but the individual values are also reported. The values were combined as the median and the data was filtered out, if there were not at least two data points or if the standard deviation exceeded a value of 1.41. All modified cysteines were filtered out if they were not quantified for at least one condition. These are the final ratios that are reported. The information on the "Gene Name" and "Name" was linked back from the FASTA database.

Downstream Analysis

For the competitive experiments, all conditions were further analyzed using Perseus software.[9] All individual values for each modified cysteine for the same condition were loaded into Perseus and analyzed using a one-sample t-test against a value of $\log_2(R) = 0$. Conditions with $p < 0.05$ were considered significant and compounds were deemed to engage a certain cysteine at a specific condition and called a "hit" if the statistical significance was $p < 0.05$ and the median ratio was $\log_2(R) > 2$ according to the values described under "Data Processing".

Analysis of Protein Essentiality

The data for the database of essential genes (DEG)[10] was used. It was downloaded from aureowiki (aureowiki.med.uni-greifswald.de). The information was linked to the information in the FASTA database through the "ordered locus name" that is reported in aureowiki and in UniProt.[11]

Analysis of Functional Sites

For all entries in the FASTA database for *S. aureus* NCTC8325 the information in the categories "Active site", "Binding Site", "DNA-binding", "Nucleotide-binding", "Site" and "Metal-binding" was downloaded from uniprot.org.[11] This information gives all amino acid residues that are in the respective functional sites. It was determined for all cysteines, whether they are in these functional sites or less than six amino acid residues in the primary sequence away from a residue in a functional site. If this is the case for any kind of functional site, the cysteine is considered as being at a functional site.

Analysis of Functional Protein Classes

Gene Ontology terms[12] for all entries in the FASTA database for *S. aureus* NCTC8325 in the category "GO - Molecular function" were downloaded from uniprot.org.[11] For each term that was present in the database at least once, a functional class was annotated manually. In this way, each term for each protein was assigned to a functional class. If a protein was only associated with terms from one functional class, it was assigned to that functional class. If a protein was associated with terms from different functional classes, the functional class of the protein was assigned according to this order of priority: enzyme, modulator / scaffolding / adaptor, receptor / transporter / channel and then gene expression / nucleic acid-binding. If no functional class was assigned to any of the terms, the protein was classified as "not assigned".

5.8 Global analysis of protein expression levels

Sample preparation

An overnight culture of *S. aureus* was grown in 5 mL B-medium in a plastic culture tube. After 1:100 dilution of the culture with fresh B medium, the cultures were grown for 2 h until an OD_{600} value between 0.4 and 0.6 was reached. Cultures were diluted into B-medium to give a final OD_{600} of 0.3 and then 12 mL diluted cultures were incubated with 0.6 µL **DGS** in DMSO (100 mM) or 0.6 µL DMSO as a control until the OD_{600} value reached around 3. The bacterial suspension was centrifuged at 6,000 g for 10 min at 4 °C and the supernatant was disposed. The pellet was resuspended in PBS and centrifuged at 6,000 g for 10 min at 4 °C again. The supernatant was removed and the pellets were stored at –80 °C. Pellets were resuspended in 200 µL 100 mM Tris pH 7.4 (lysis buffer) and lysed by sonication with 5 times, 20 sec pulses at 80% max. power on ice. 75 µL solution of 10% (w/v) SDS and 1.25% (w/v) sodium deoxycholate in lysis buffer was added and samples heated for 10 min at 90 °C. Lysates were sonicated for 10 s at 10% intensity to shear nucleic acids and centrifuged at 10,000 g for 15 min at 4 °C to pellet debris. Protein concentration of the lysate was determined using a bicinchoninic acid (BCA) assay and the concentration was adjusted to 0.5 mg/mL with PBS. Subsequently, proteins were precipitated with 4 mL of cold acetone (−80 °C, MS grade) overnight at –80 °C. The precipitated proteins were thawed on ice, pelletized (12,000 g, 20 min, 4 °C) and the supernatant was disposed. Proteins were washed three times with 1 mL cold methanol (–80 °C, MS grade). Resuspension was achieved by sonication (10 sec at 10% intensity) and proteins

were pelletized via centrifugation (12,000 g, 20 min, 4 °C). The following steps from digestion to filtering, were performed as described for the conventional ABPP approach.

MS measurement

Samples were analyzed *via* HPLC-MS/MS using an UltiMate 3000 nano HPLC system (Dionex, Sunnyvale, California, USA) equipped with Acclaim C18 PepMap100 75 µm ID x 2 cm trap and Acclaim C18 PepMap RSLC, 75 µM ID x 15 cm separation columns coupled to Thermo Fischer LTQ Orbitrap Fusion (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Samples were loaded on the trap and washed for 10 min with 0.1% FA (at 5 µL/min), then transferred to the analytical column and separated using a, 112 min gradient from 4% to 35% MeCN followed by 4 min at 80% MeCN in 0.1% FA (at 200 nL/min flow rate). The LTQ Orbitrap Fusion was operated in a 3 second top speed data dependent mode. Full scan acquisition was performed in the orbitrap at a resolution of 120,000 and an ion target of 4e5 in a scan range of 300 – 1700 m/z. Monoisotopic precursor selection as well as dynamic exclusion for 60 sec were enabled. Precursors with charge states of 2 – 7 and intensities greater than 5e3 were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z. Precursors were collected to a target of 1e2 for a maximum injection time of 250 ms with "inject ions for all available parallelizable time" enabled. Fragments were generated using higher-energy collisional dissociation (HCD) and detected in the ion trap at a rapid scan rate. Internal calibration was performed using the ion signal of fluoranthene cations (EASY-ETD/IC source). MS raw files were processed with MaxQuant version 1.6.0.16 as described above for conventional ABPP. Statistical analysis was performed as described above using Perseus version 1.6.2.2.

Analysis of Gene Ontology terms

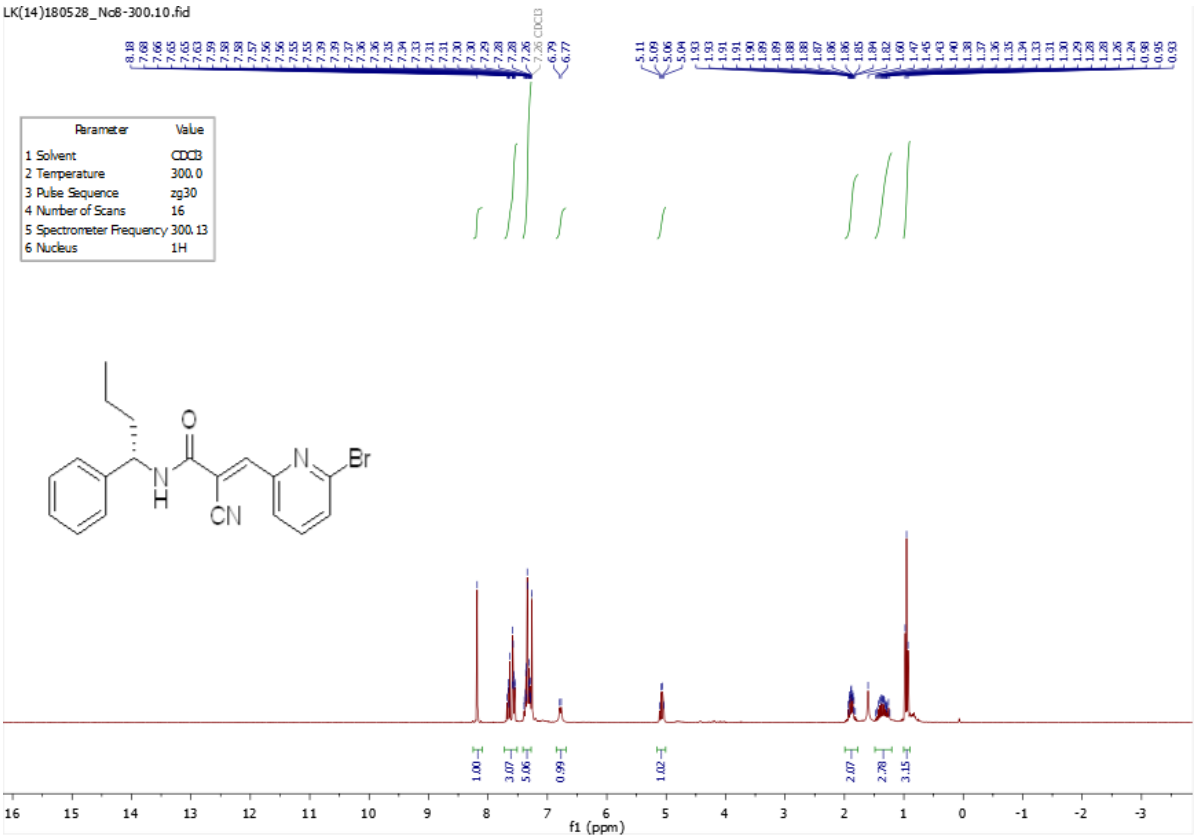
Gene Ontology terms[12] for all entries in the FASTA database for *S. aureus* NCTC8325 in the category "GO – Biological Process" were downloaded from uniprot.org.[11] An enrichment analysis was performed with the BiNGO 3.0.3. app[13] in cytoscape 3.7.1.[14] using a hypergeometric test with Benjamini and Hochberg FDR correction and a significance level of 0.05. We compared the up-regulated proteins to all proteins quantified in the experiments. The terms with the highest corrected p-values are reported.

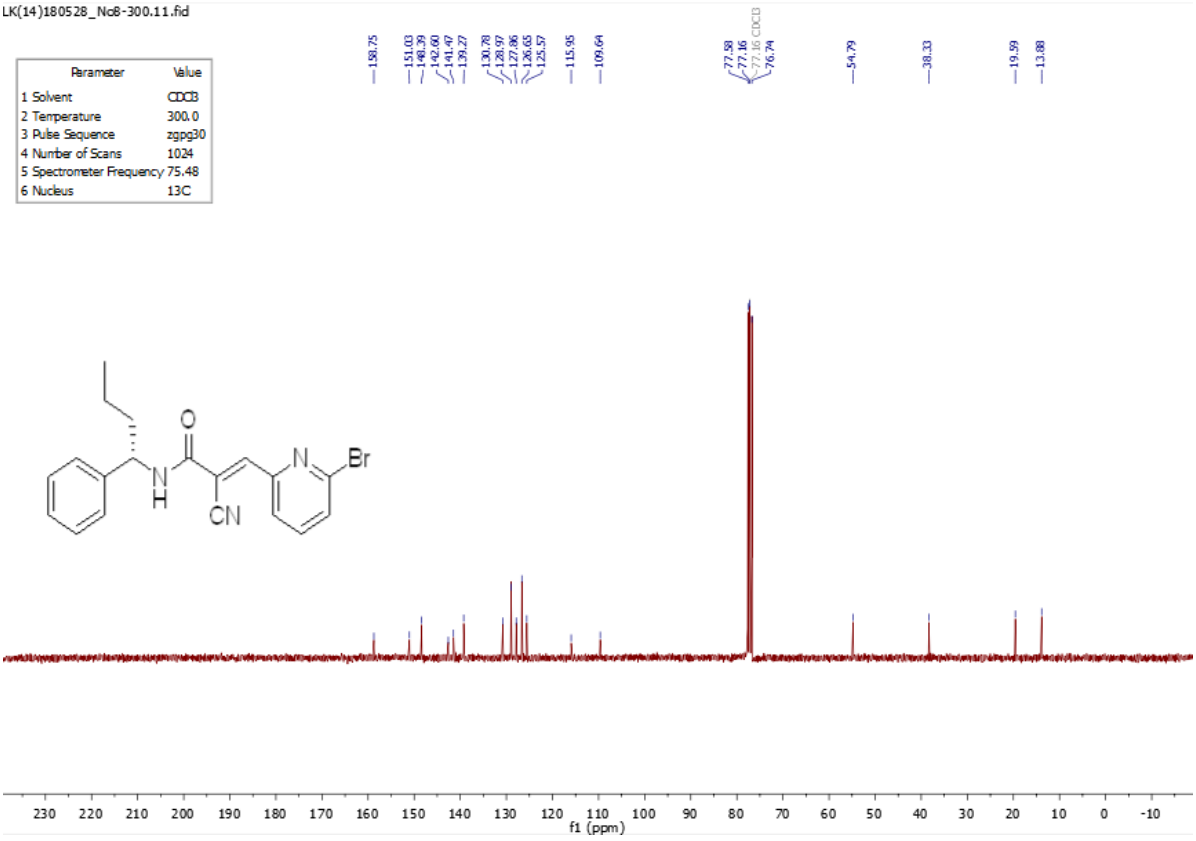
6. Literature

1. J. A. Vizcaino *et al.*, *Nat. Biotechnol.*, 2014, **32**, 223-226.
2. J. A. Vizcaino *et al.*, *Nucleic Acids Res.*, 2016, **44**, D447-456.
3. A. Iraji *et al.*, *Eur. J. Med. Chem.*, 2017, **141**, 690-702.
4. W. Ried and B. Schleimer, *Angew. Chem.*, 1958, **70**, 164-164.
5. C. V. Gomez, D. C. Cruz, R. Mose and K. A. Jorgensen, *Chem. Commun.*, 2014, **50**, 6035-6038.
6. P. Le *et al.*, *Nat. Chem.*, DOI: 10.1038/s41557-019-0378-7.
7. J. Cox and M. Mann, *Nat. Biotechnol.*, 2008, **26**, 1367-1372.
8. P. R. A. Zanon, L. Lewald and S. M. Hacker, *Angew. Chem. Int. Ed.*, DOI: 10.1002/anie.201912075.
9. S. Tyanova *et al.*, *Nat. Methods*, 2016, **13**, 731-740.
10. R. R. Chaudhuri *et al.*, *BMC Genomics*, 2009, **10**, 291.
11. The UniProt Consortium, *Nucleic Acids Res.*, 2019, **47**, D506-D515.
12. P. Gaudet, M. S. Livstone, S. E. Lewis and P. D. Thomas, *Brief. Bioinform.*, 2011, **12**, 449-462.
13. S. Maere, K. Heymans and M. Kuiper, *Bioinformatics*, 2005, **21**, 3448-3449.
14. P. Shannon *et al.*, *Genome Res.*, 2003, **13**, 2498-2504.

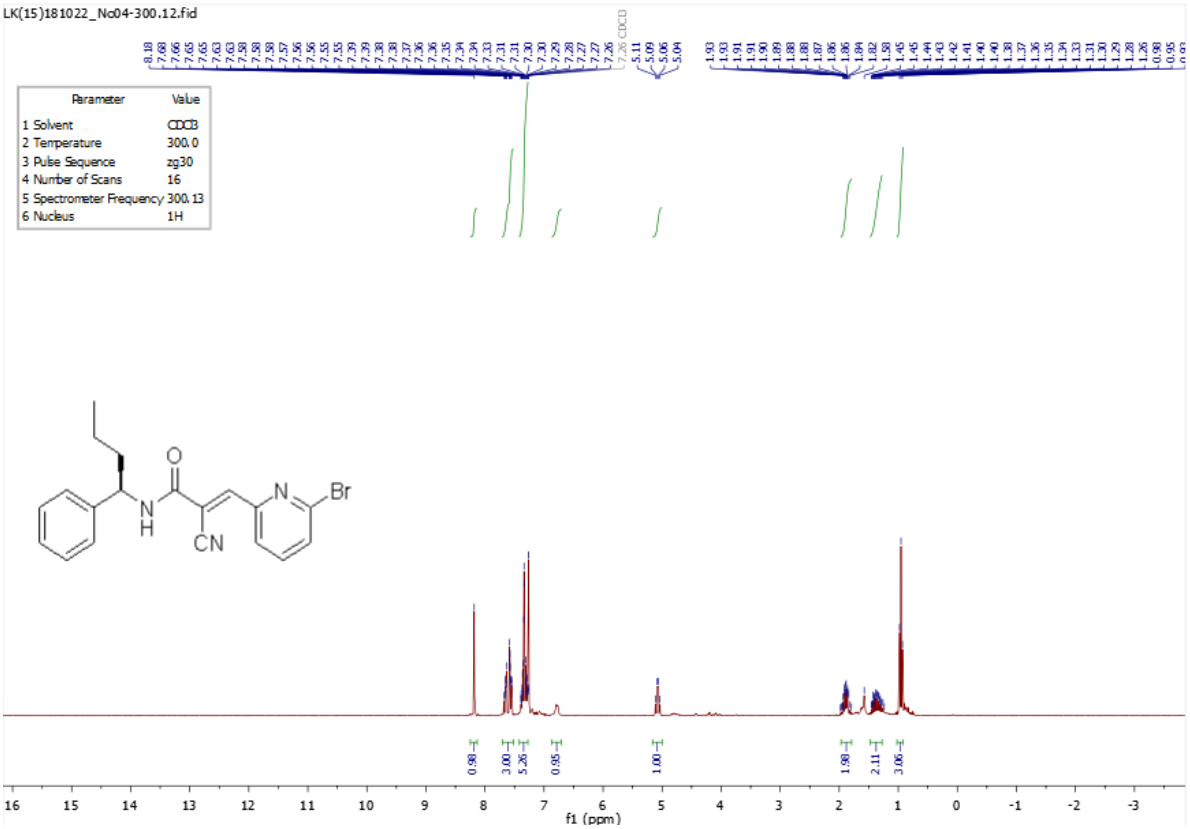
7. NMR spectra

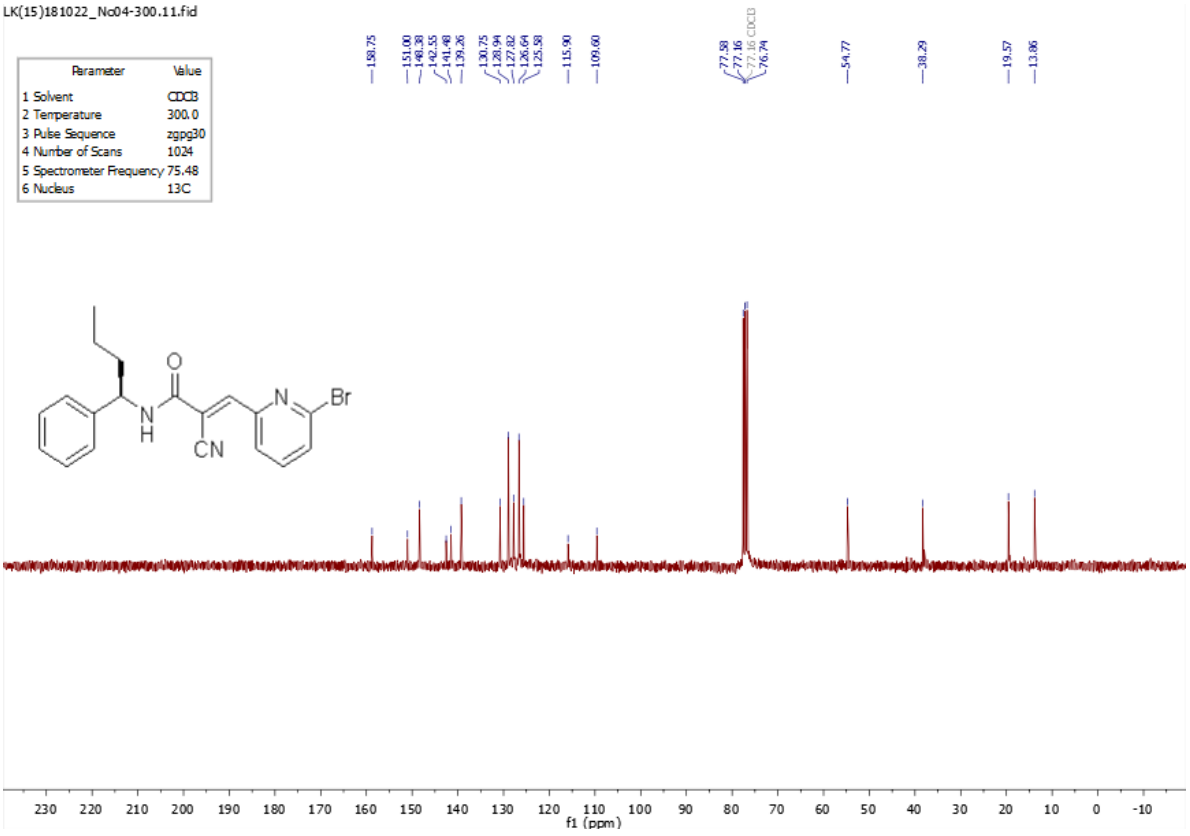
(*S*,*E*)-3-(6-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (DGS)



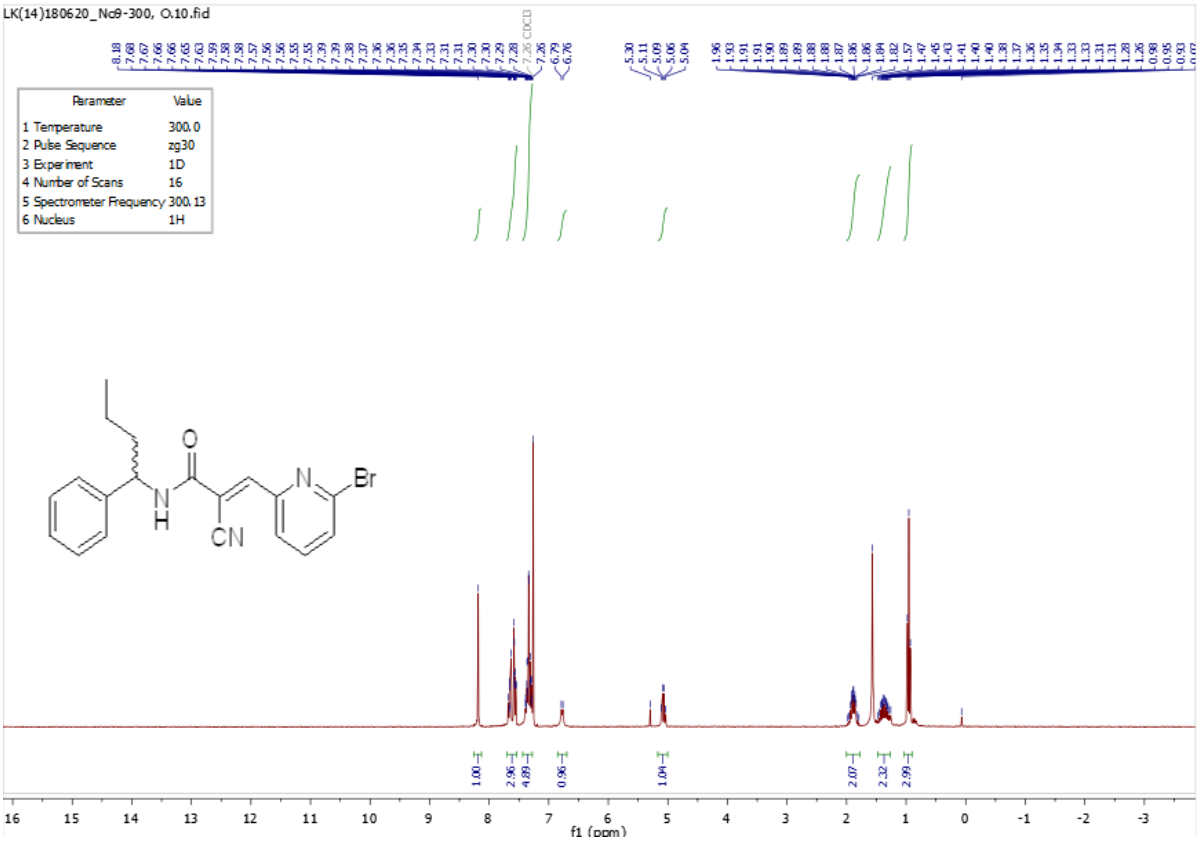


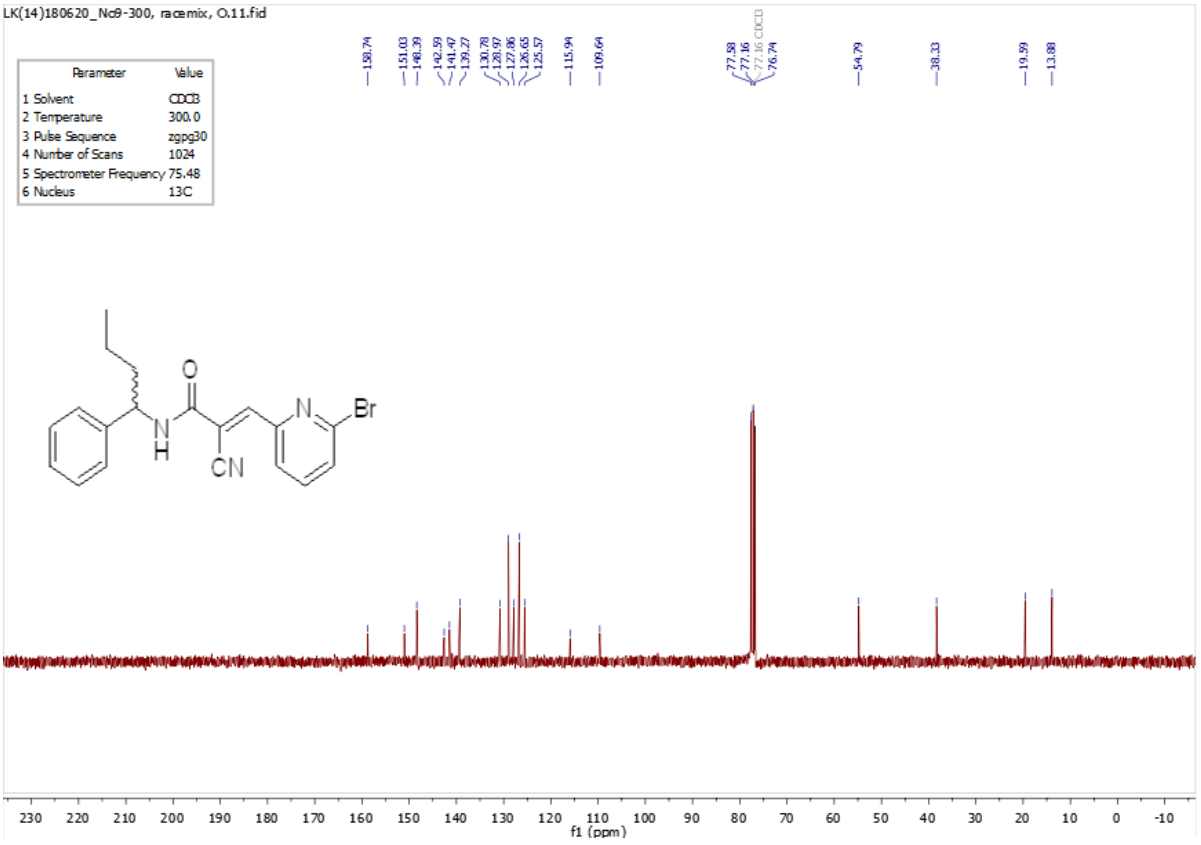
(*R*,*E*)-3-(6-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide ((*R*)-DGS)



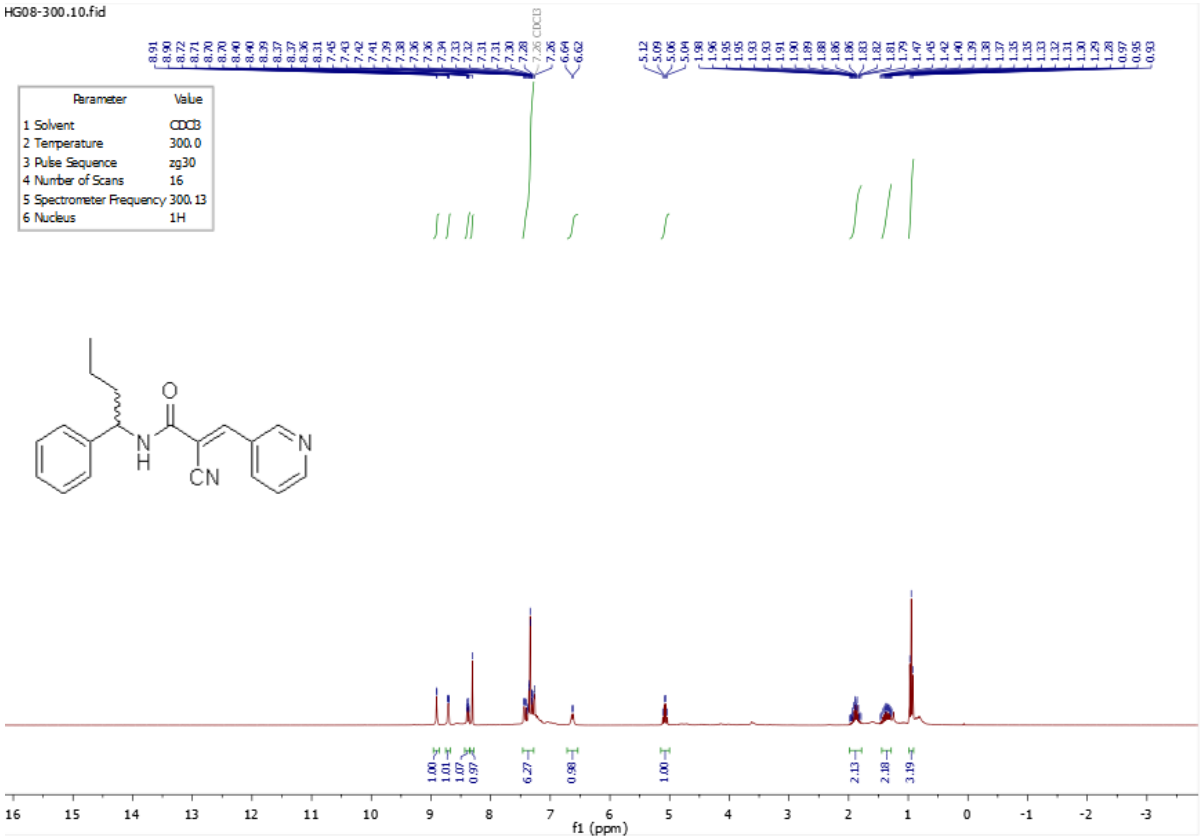


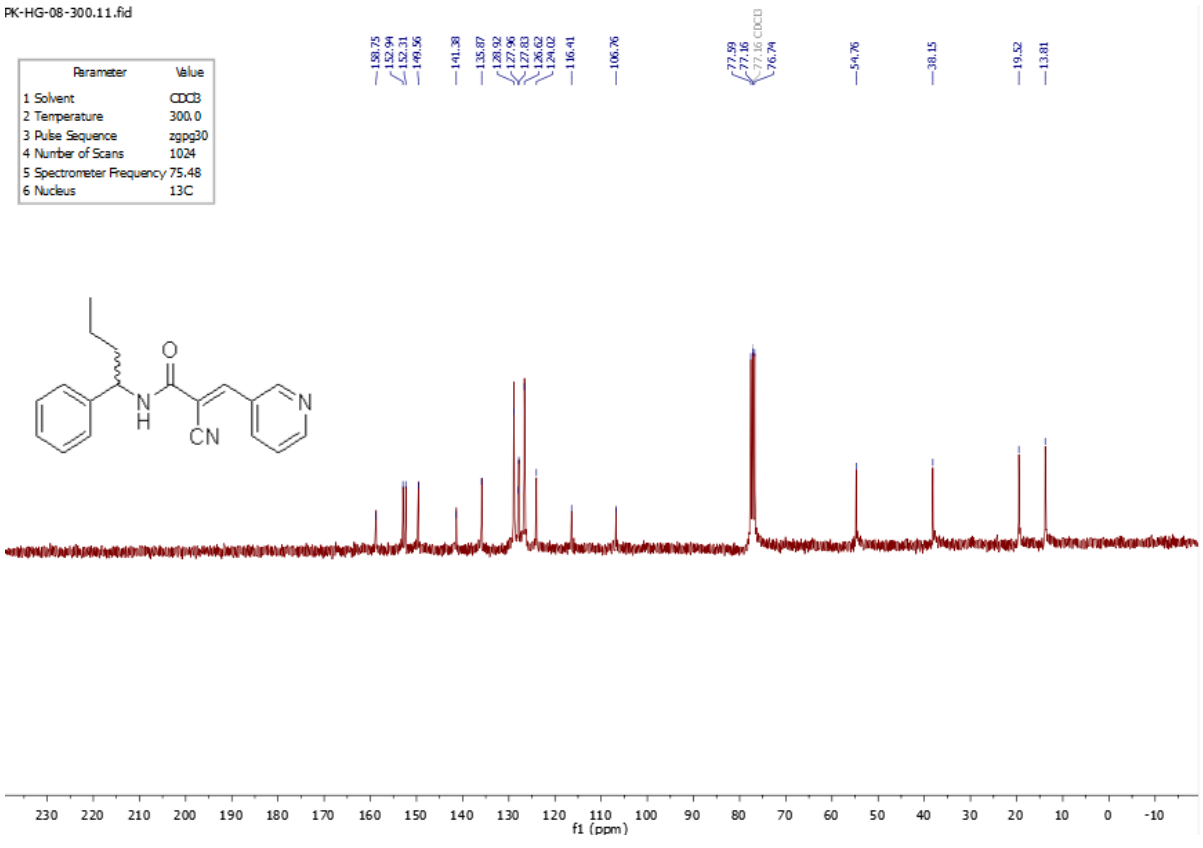
(*E*)-3-(6-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide ((*R*,*S*)-DGS)



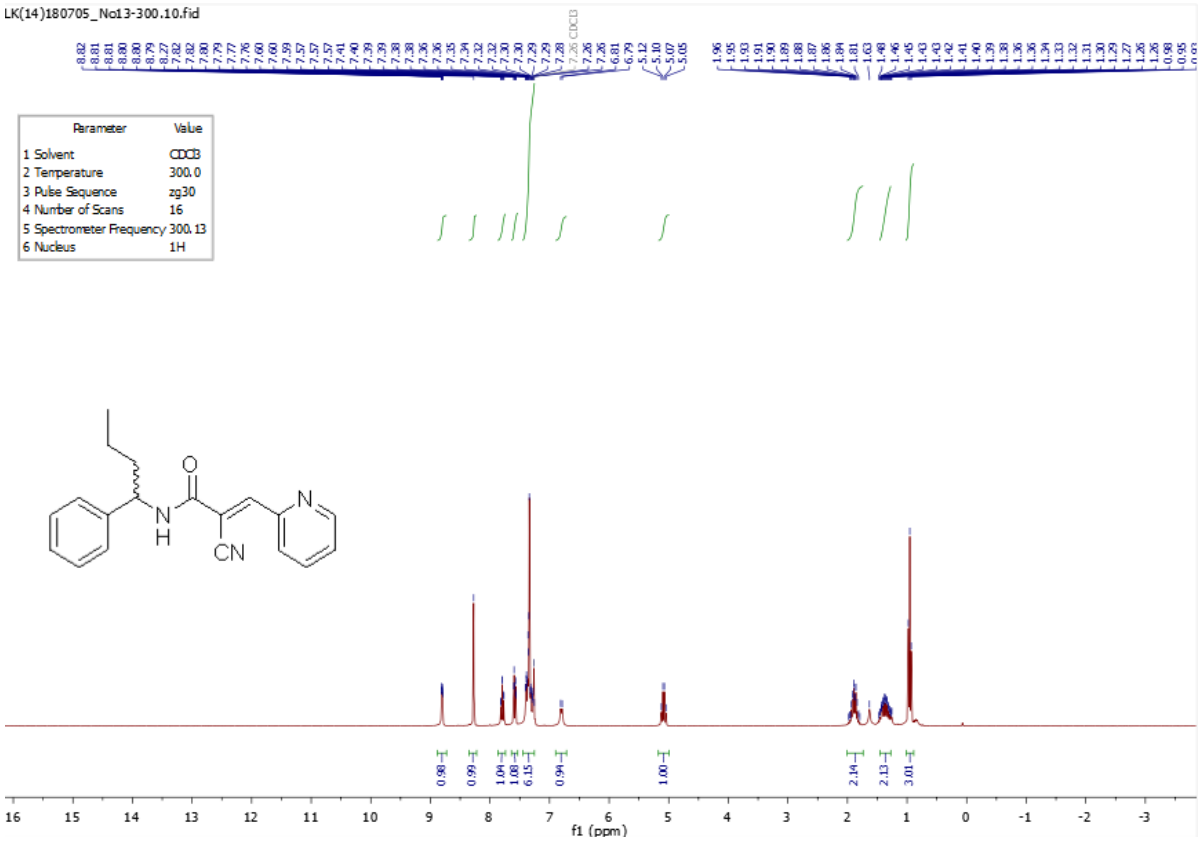


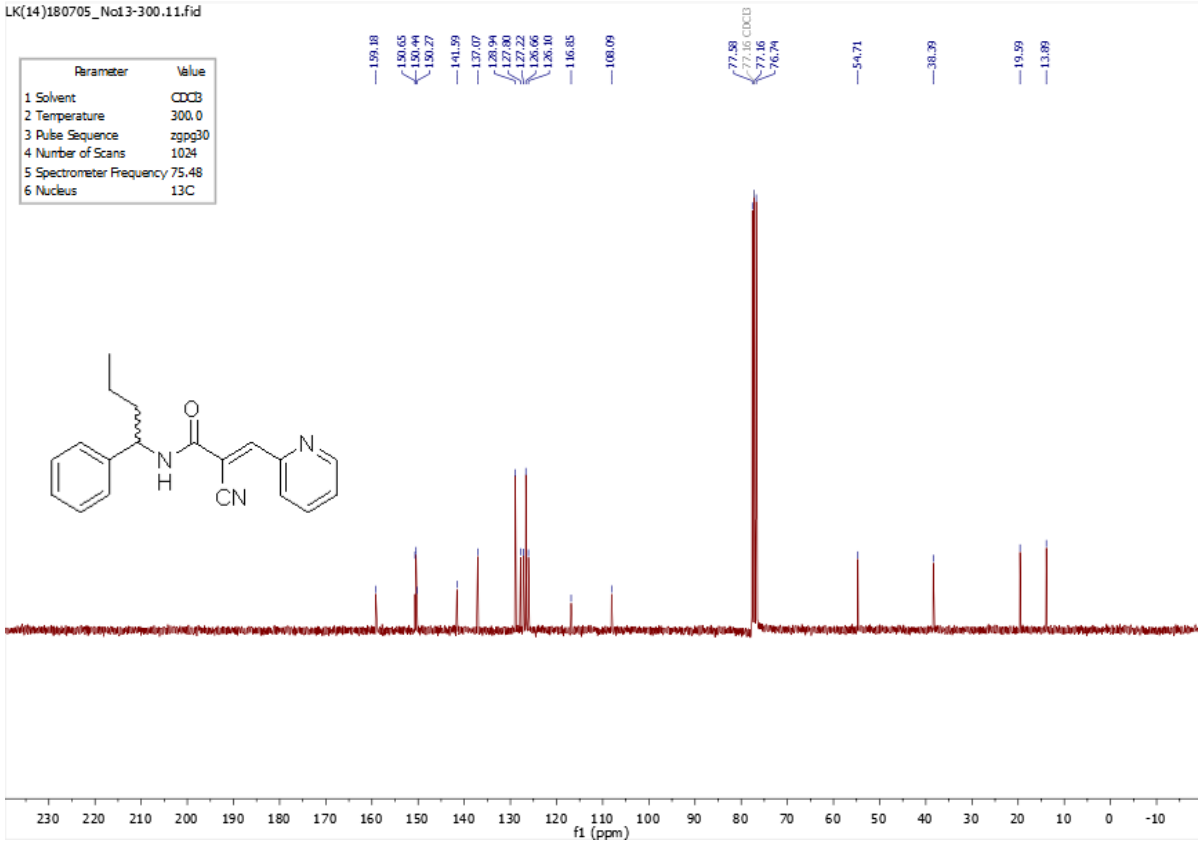
(*E*)-2-Cyano-*N*-(1-phenylbutyl)-3-(pyridin-3-yl)acrylamide (1)



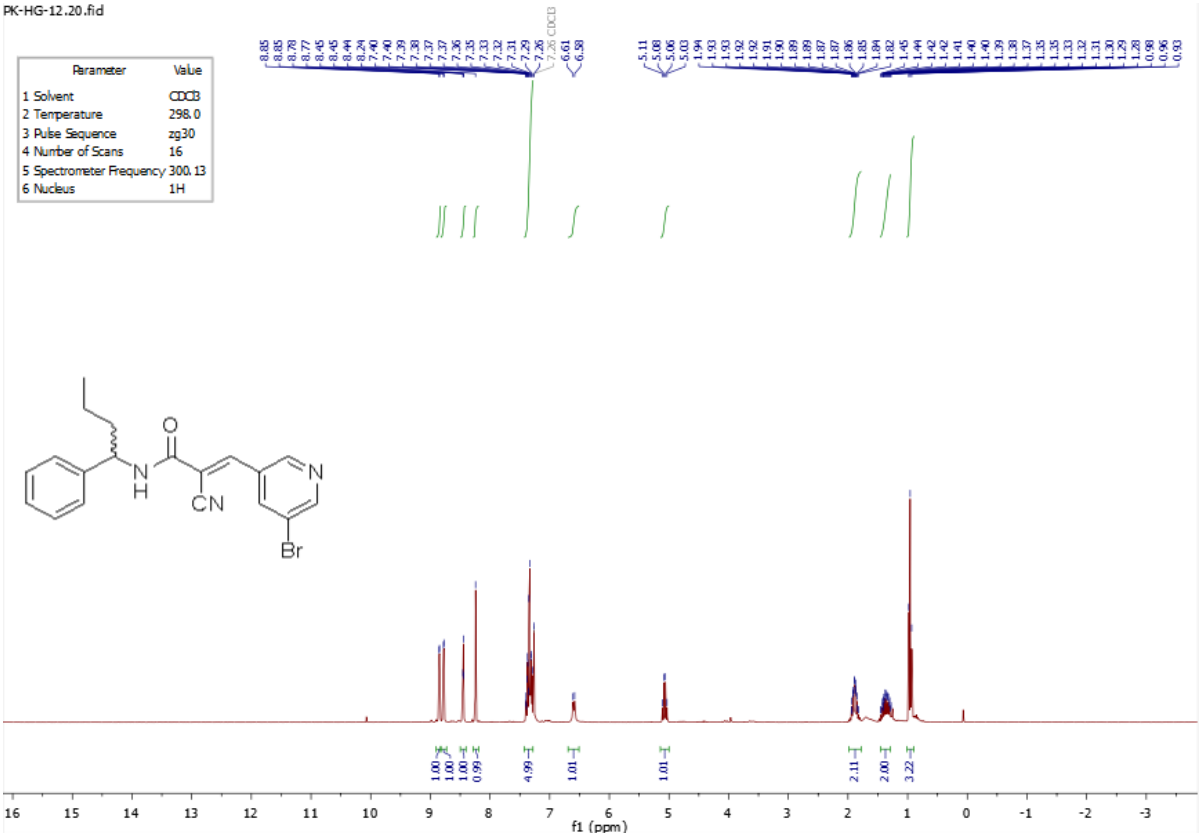


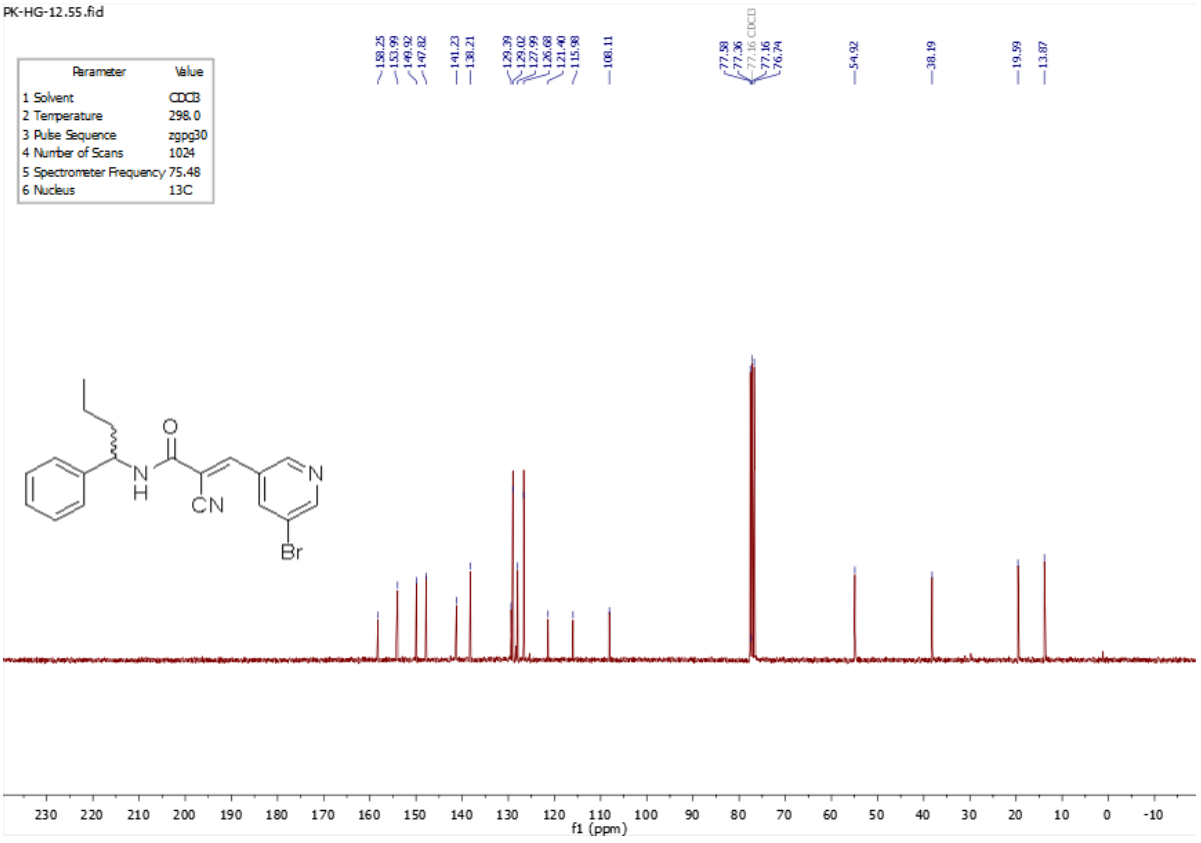
(*E*)-2-Cyano-*N*-(1-phenylbutyl)-3-(pyridin-2-yl)acrylamide (**2**)



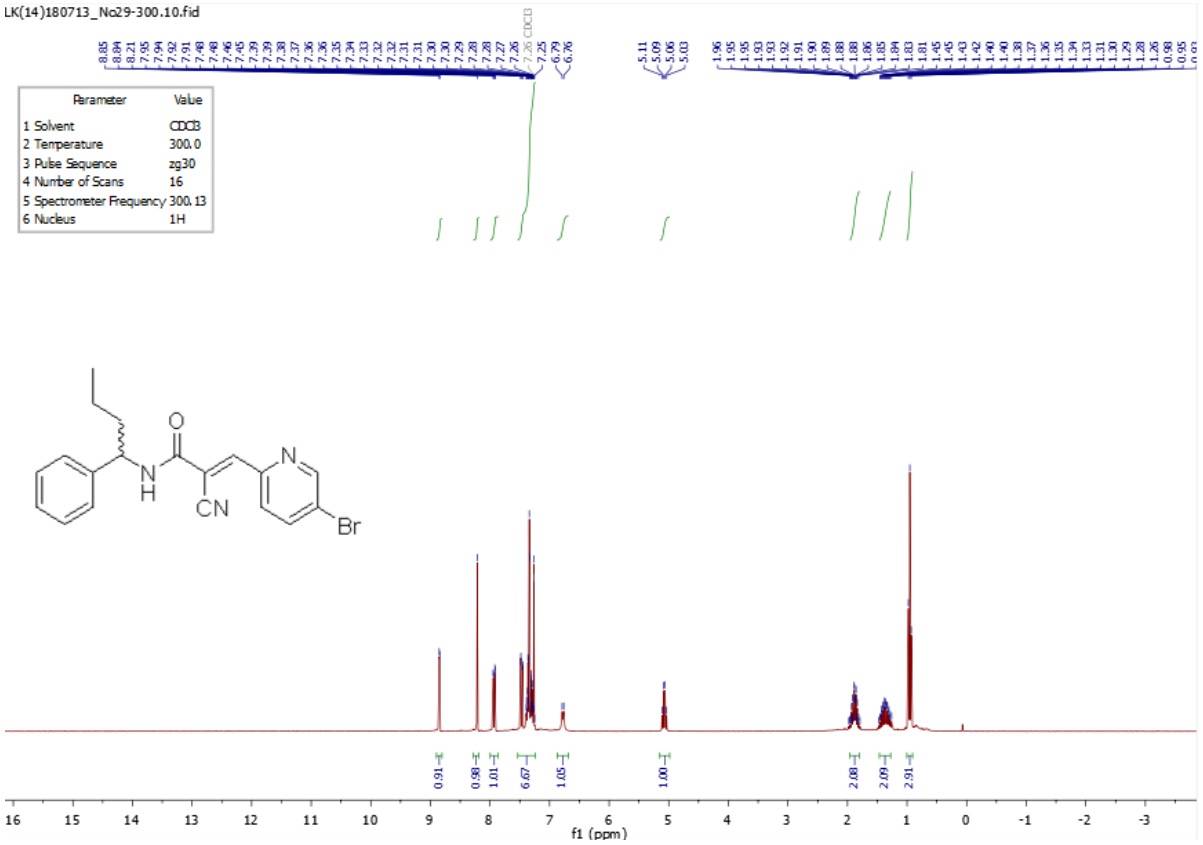


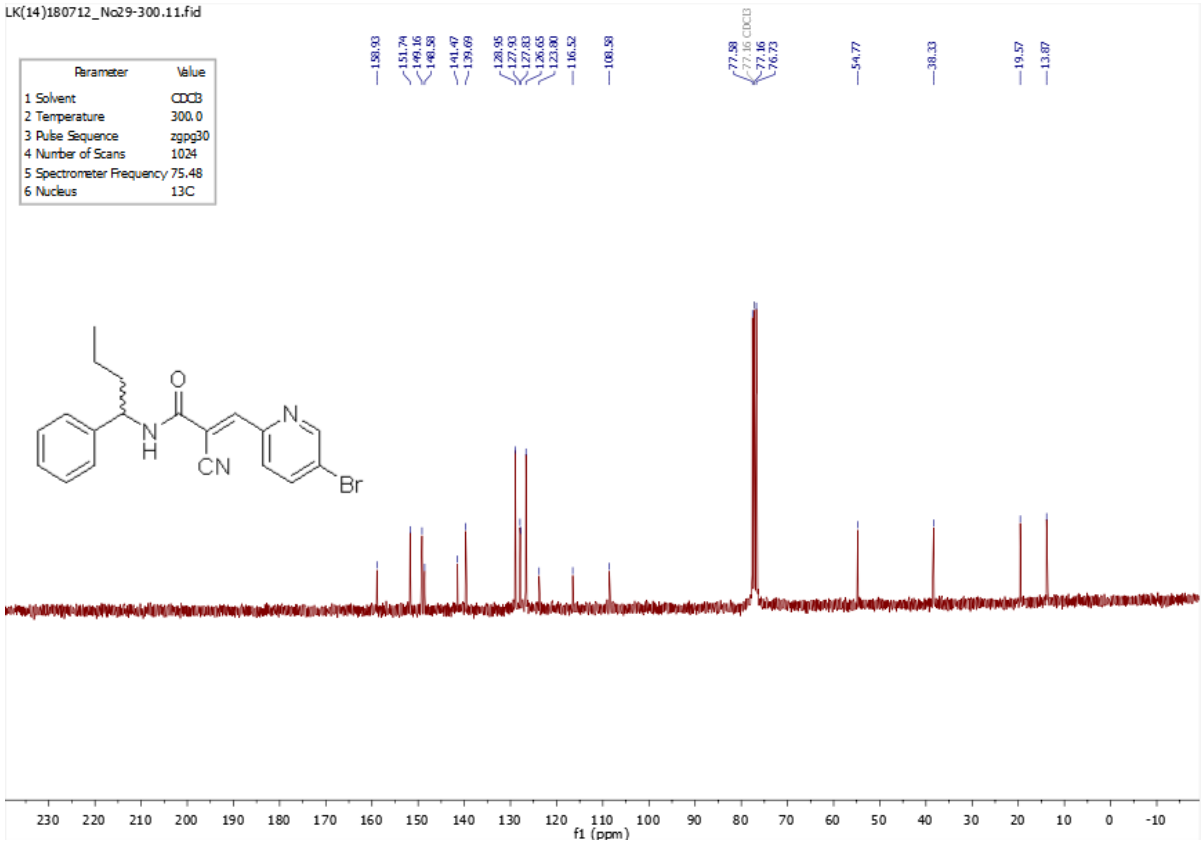
(*E*)-3-(5-Bromopyridin-3-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (3)



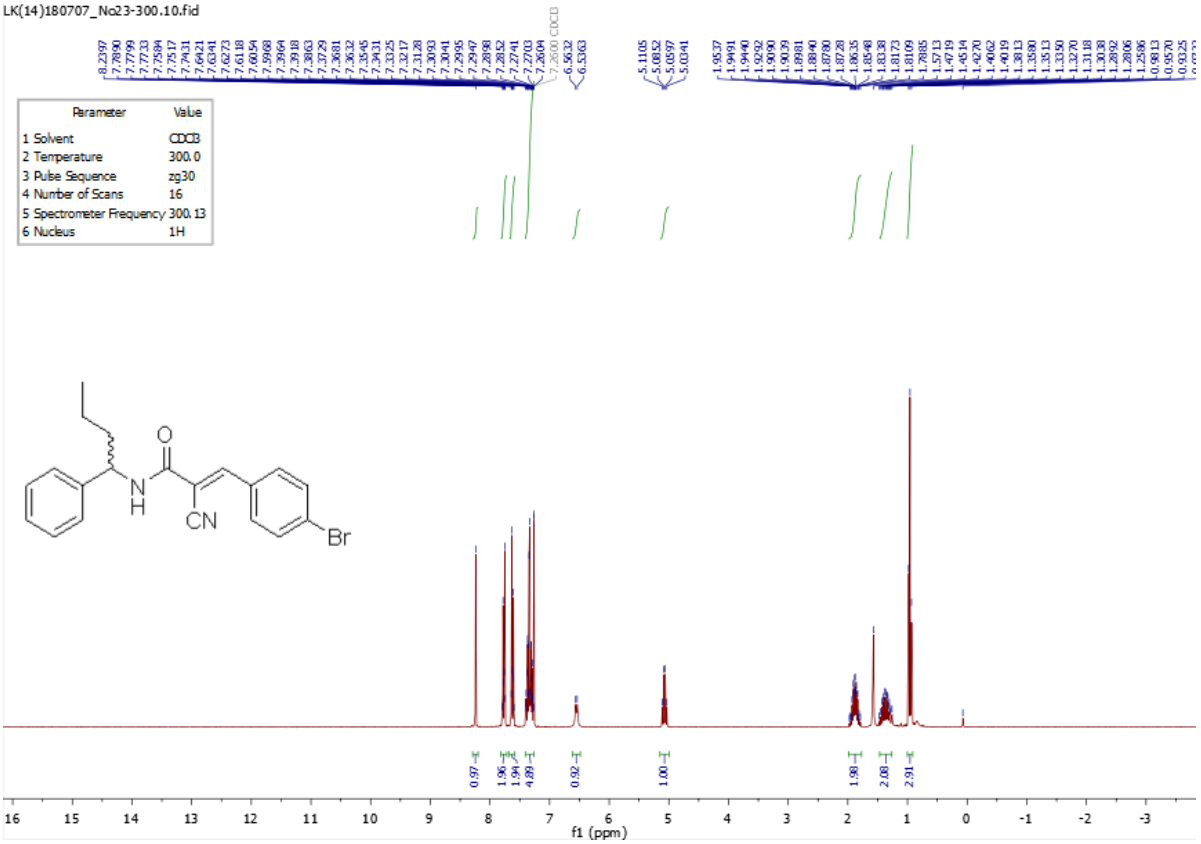


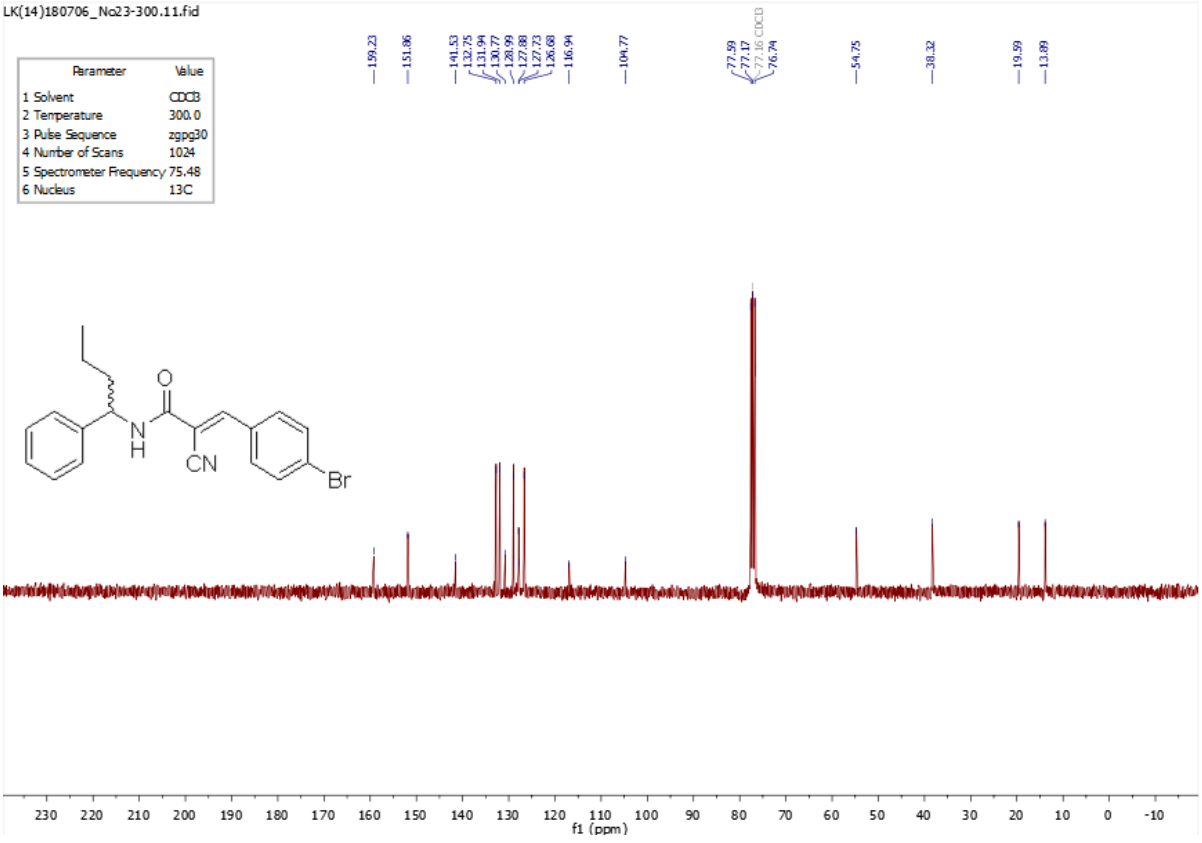
(*E*)-3-(5-Bromopyridin-2-yl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (4)



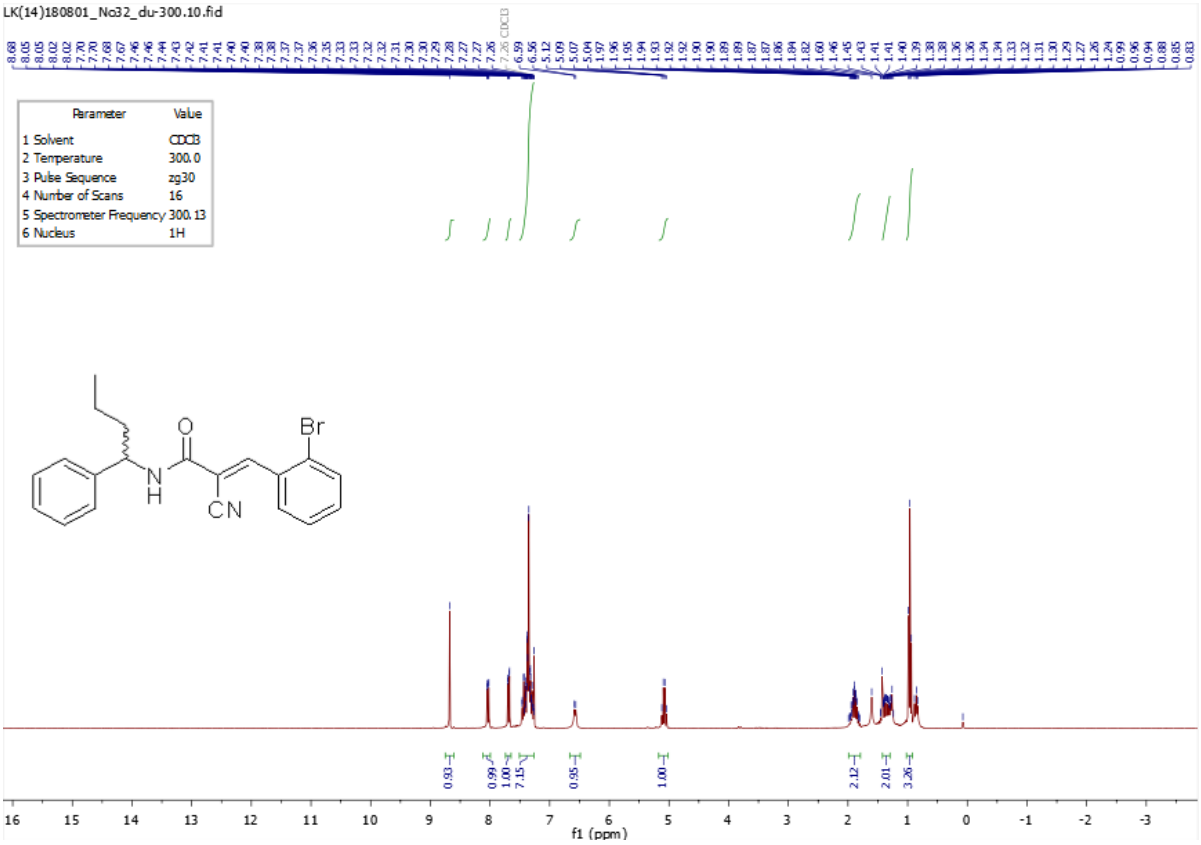


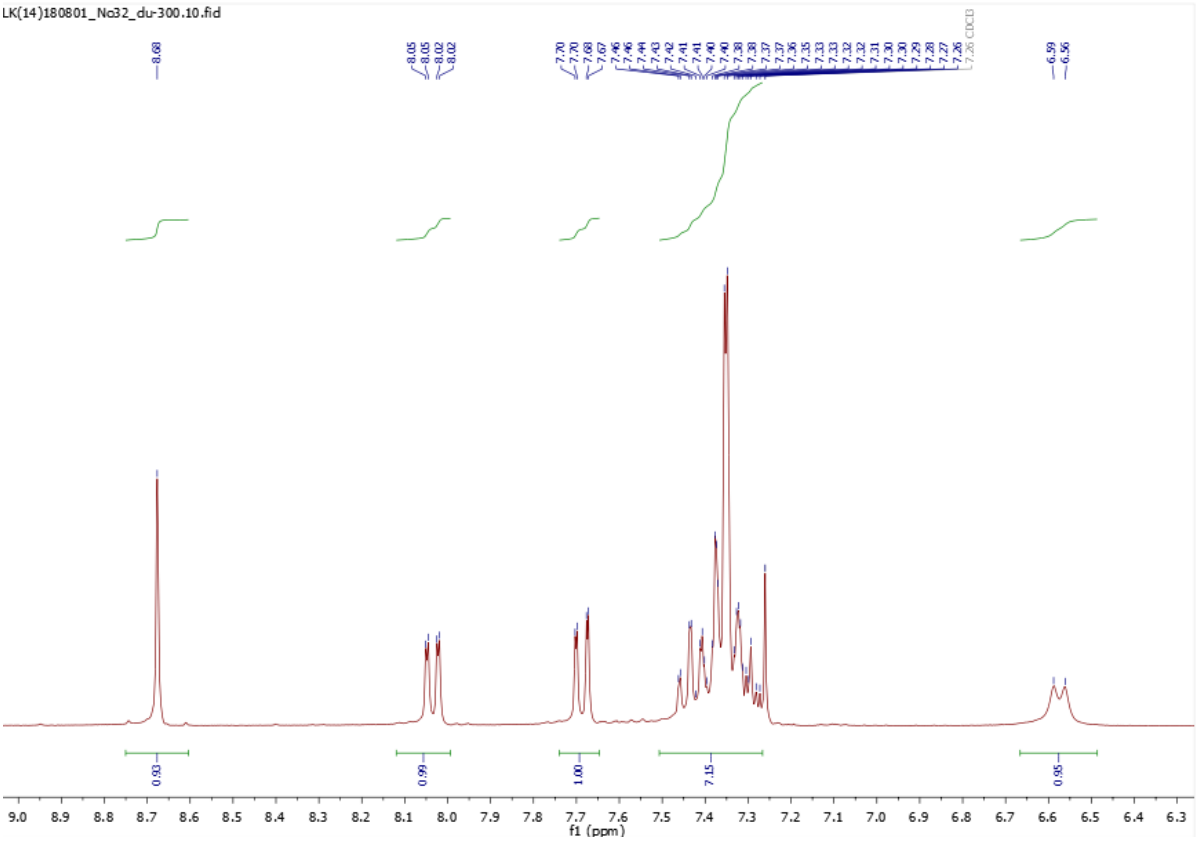
(*E*)-3-(4-Bromophenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (5)

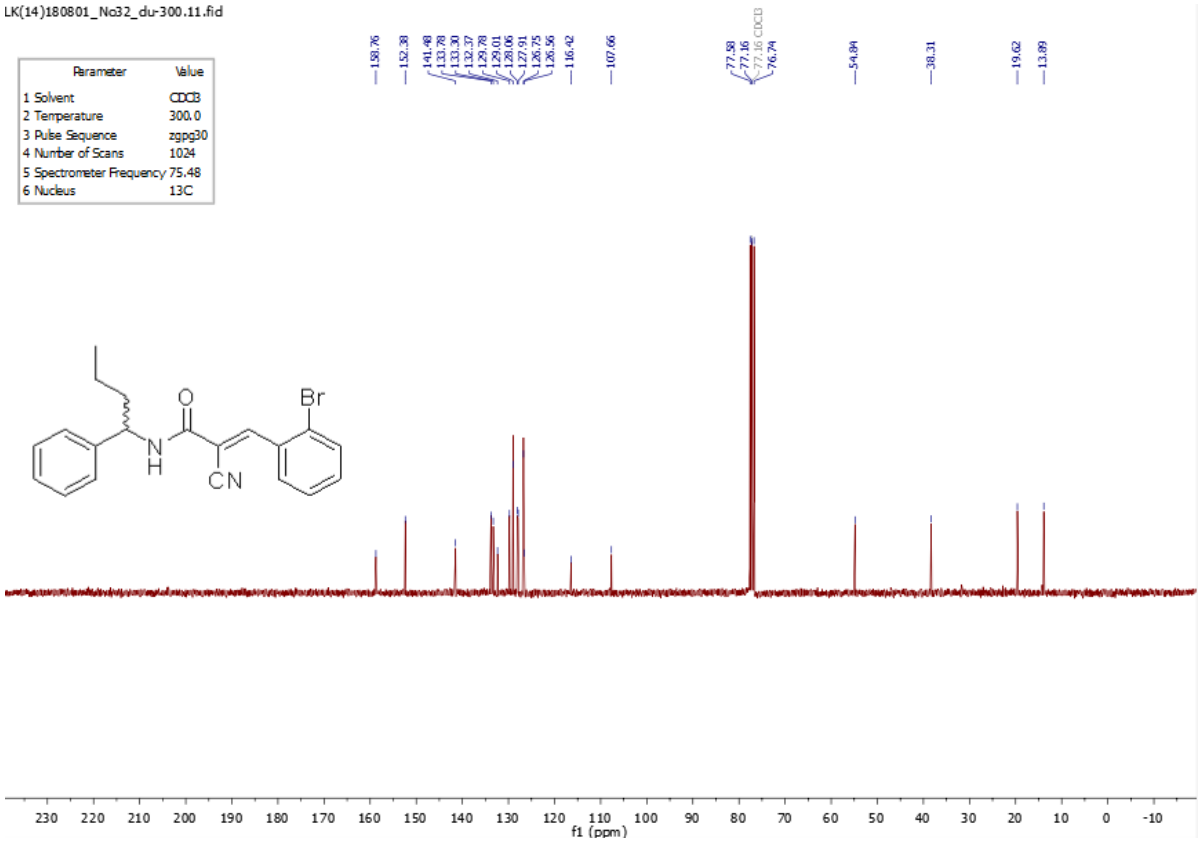




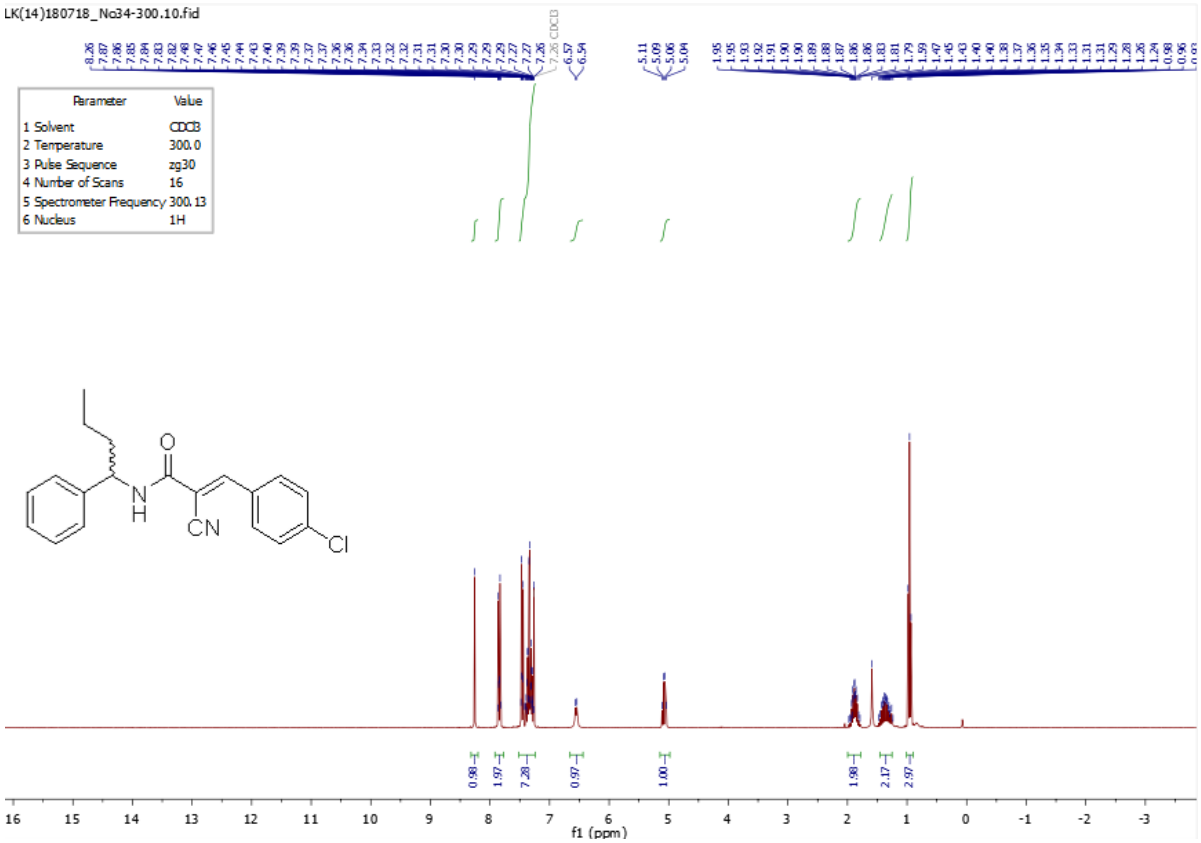
(*E*)-3-(2-Bromophenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (6)

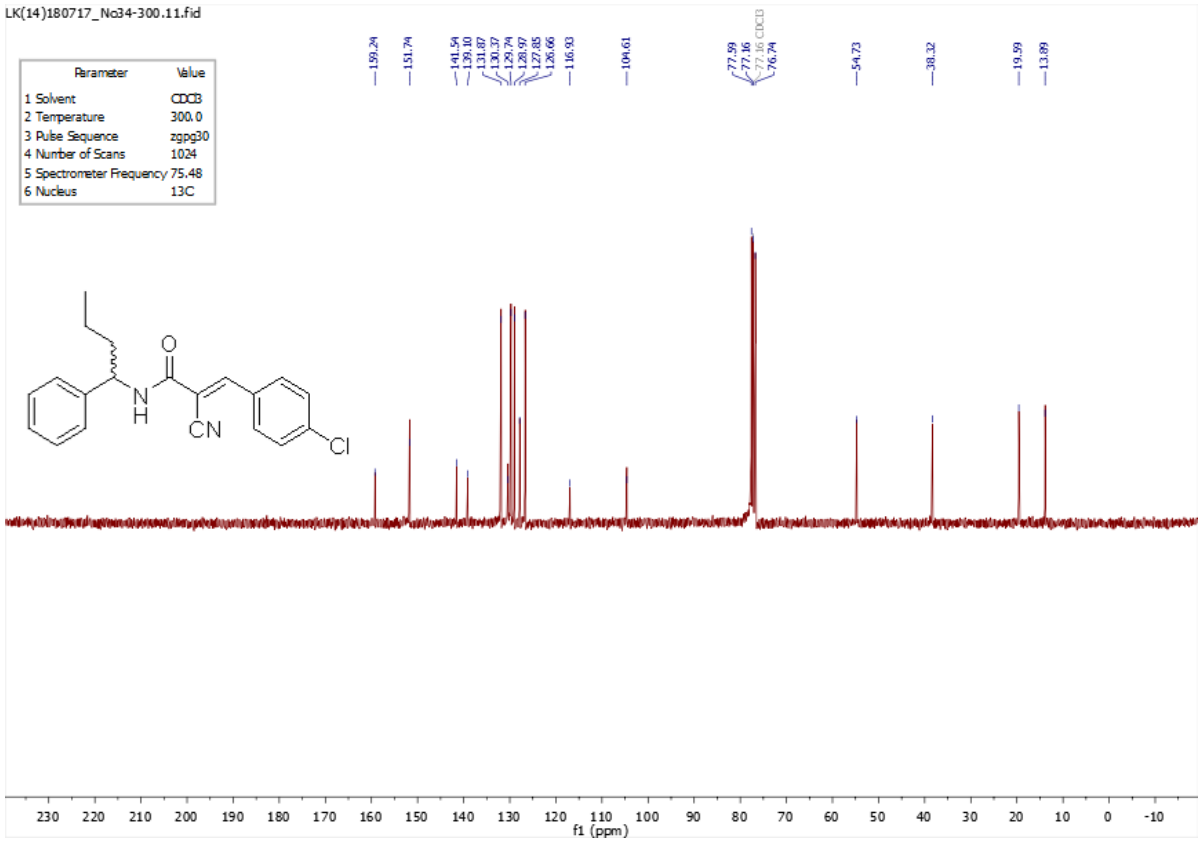




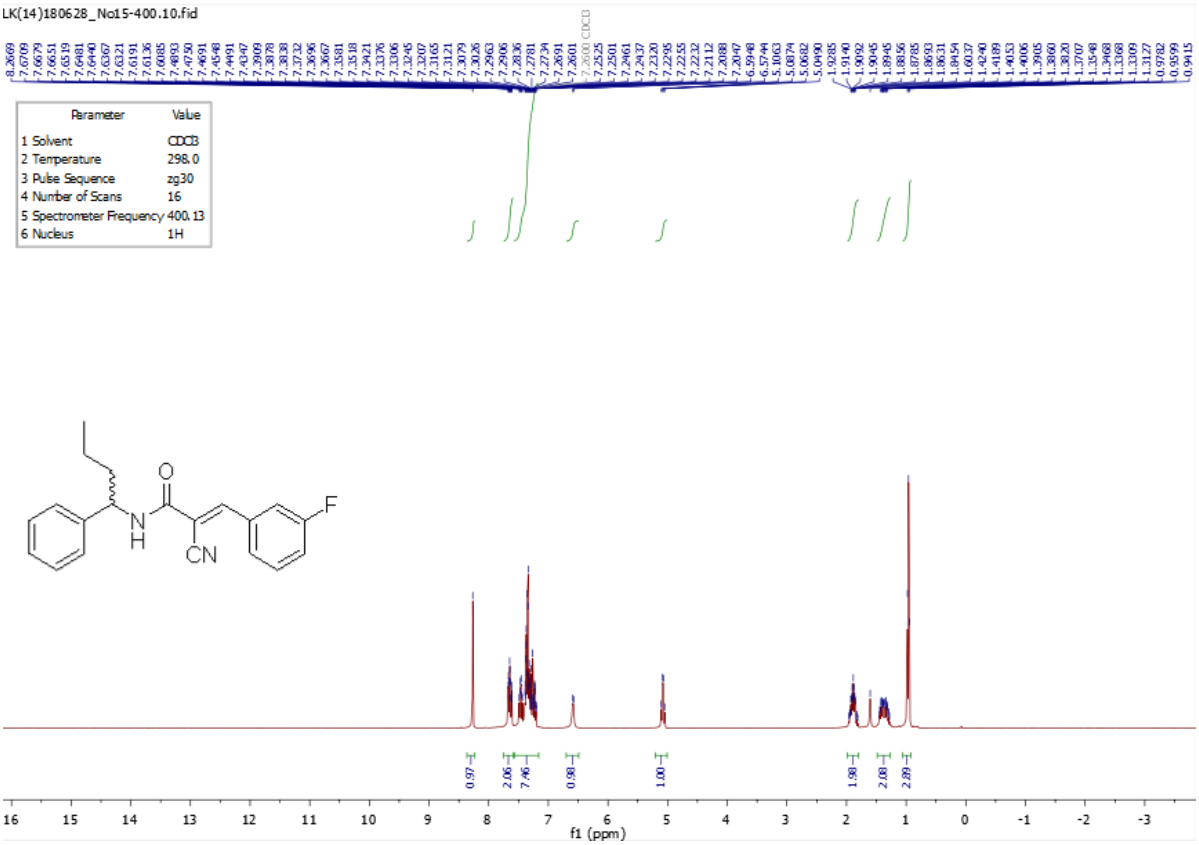

LK(14)180801_Nc32_du-300.11.fid
Parameter Value
1 Solvent CDCl3
2 Temperature 300.0
3 Pulse Sequence zgpg30
4 Number of Scans 1024
5 Spectrometer Frequency 75.48
6 Nucleus 13C
158.76
152.38
141.48
133.78
133.30
132.37
129.78
129.01
128.06
127.91
126.75
126.56
116.42
107.66
77.58
77.16
77.16 CDCl3
76.74
54.84
38.31
19.62
13.89
O
Br
N
H
CN
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

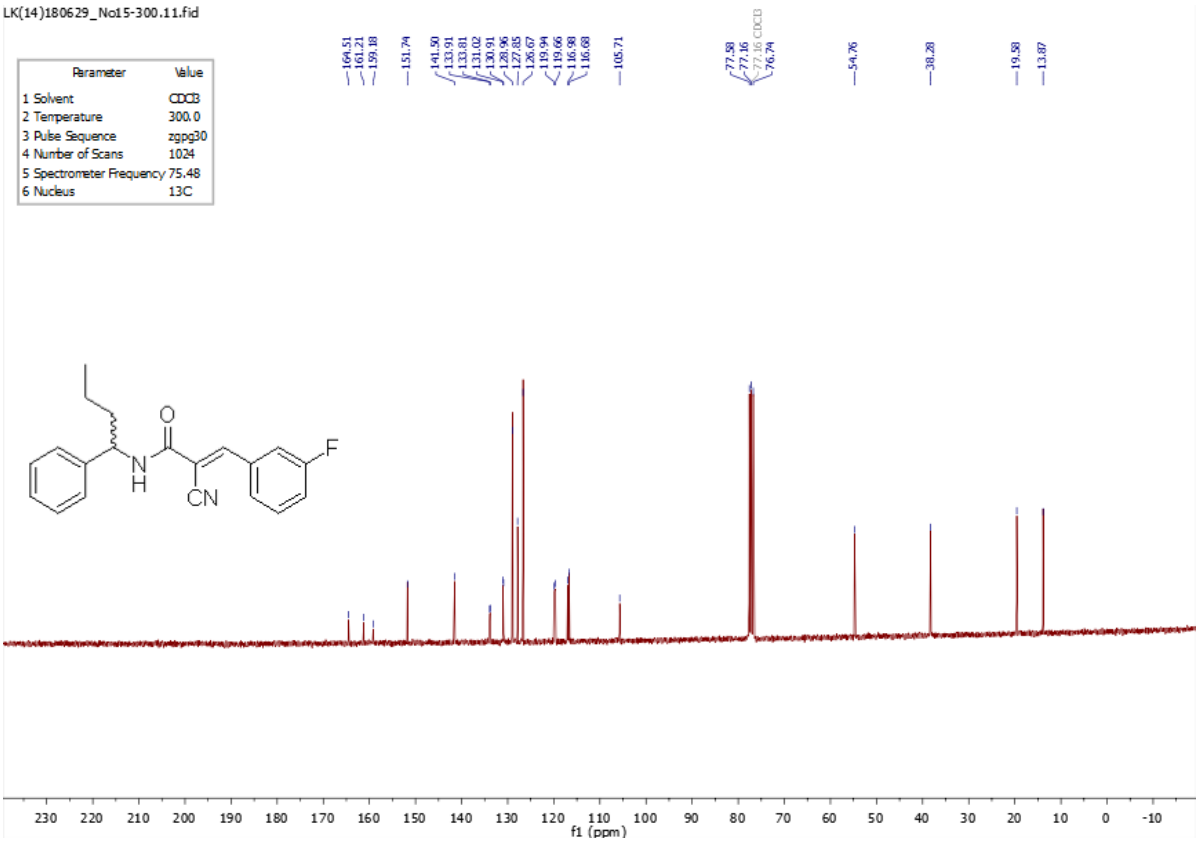
(*E*)-3-(4-Chlorophenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (**7**)



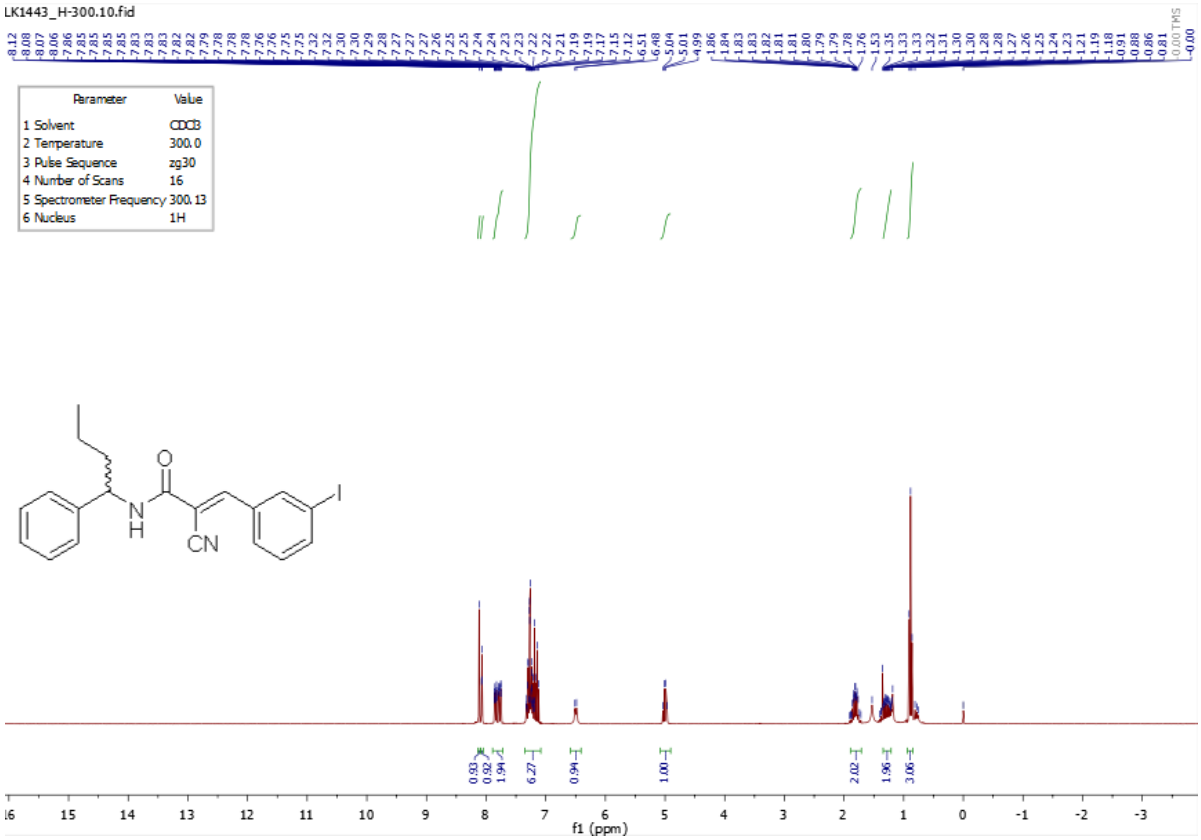


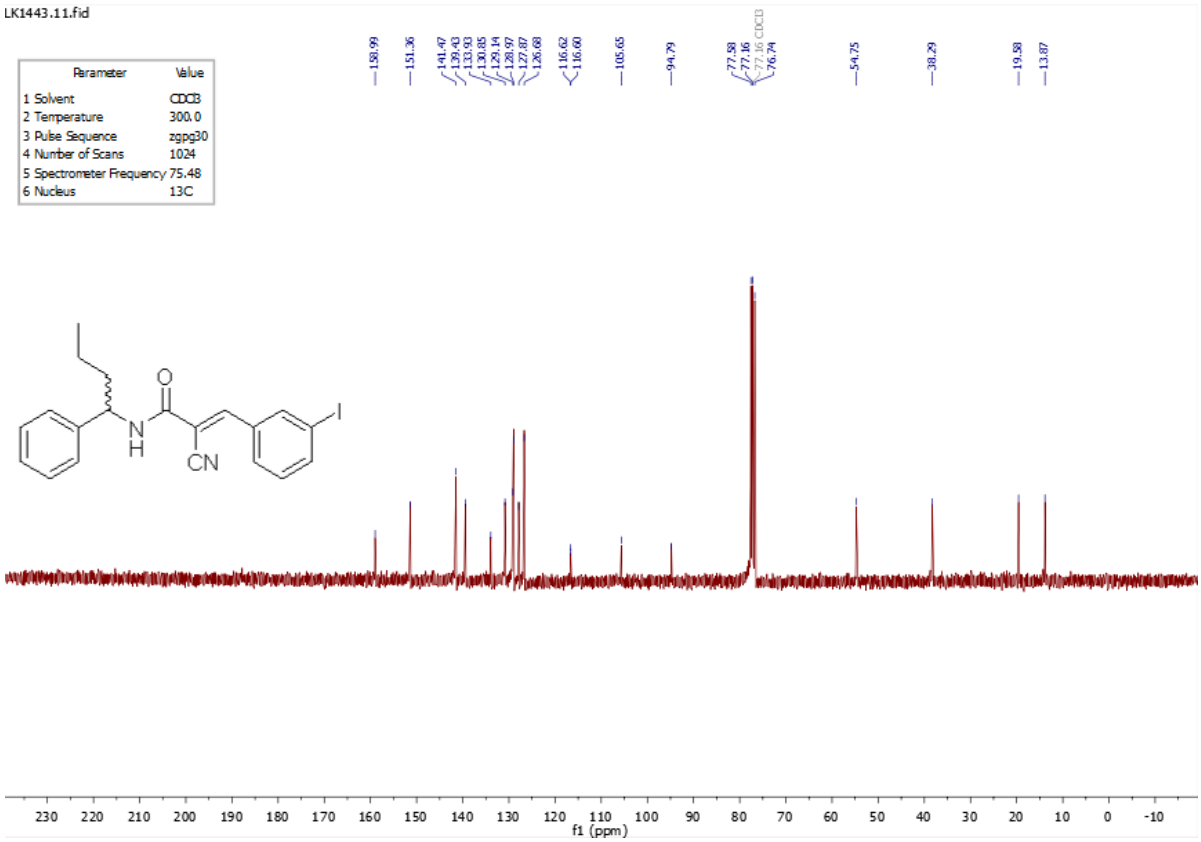
(*E*)-2-Cyano-3-(3-fluorophenyl)-*N*-(1-phenylbutyl)acrylamide (8)



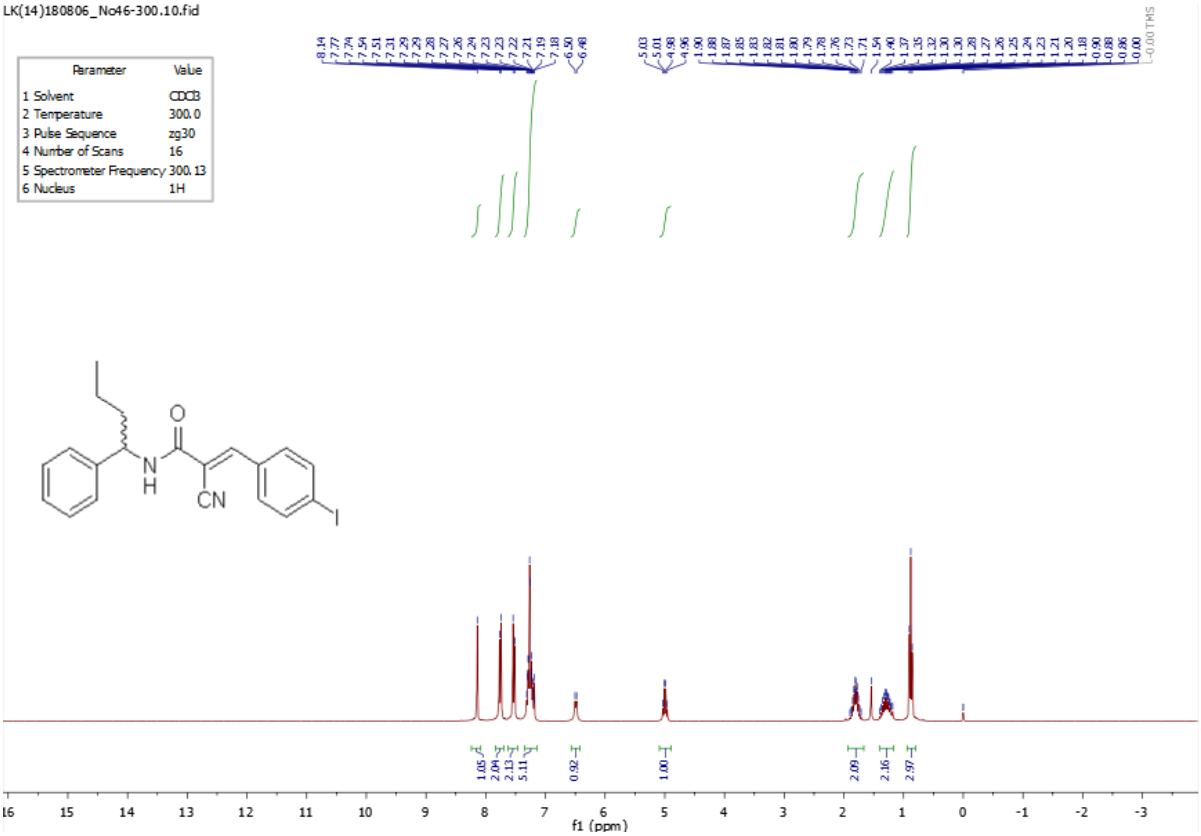


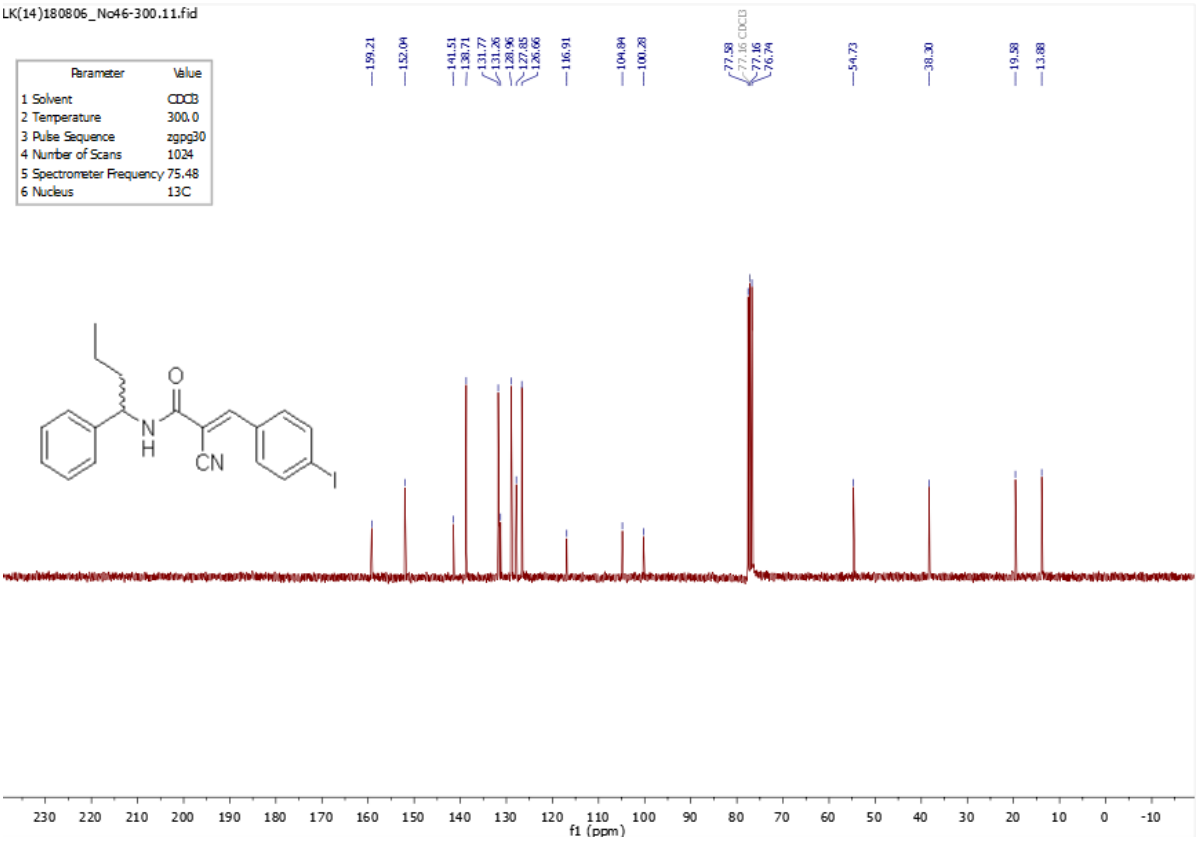
(*E*)-2-Cyano-3-(3-iodophenyl)-*N*-(1-phenylbutyl)acrylamide (**9**)



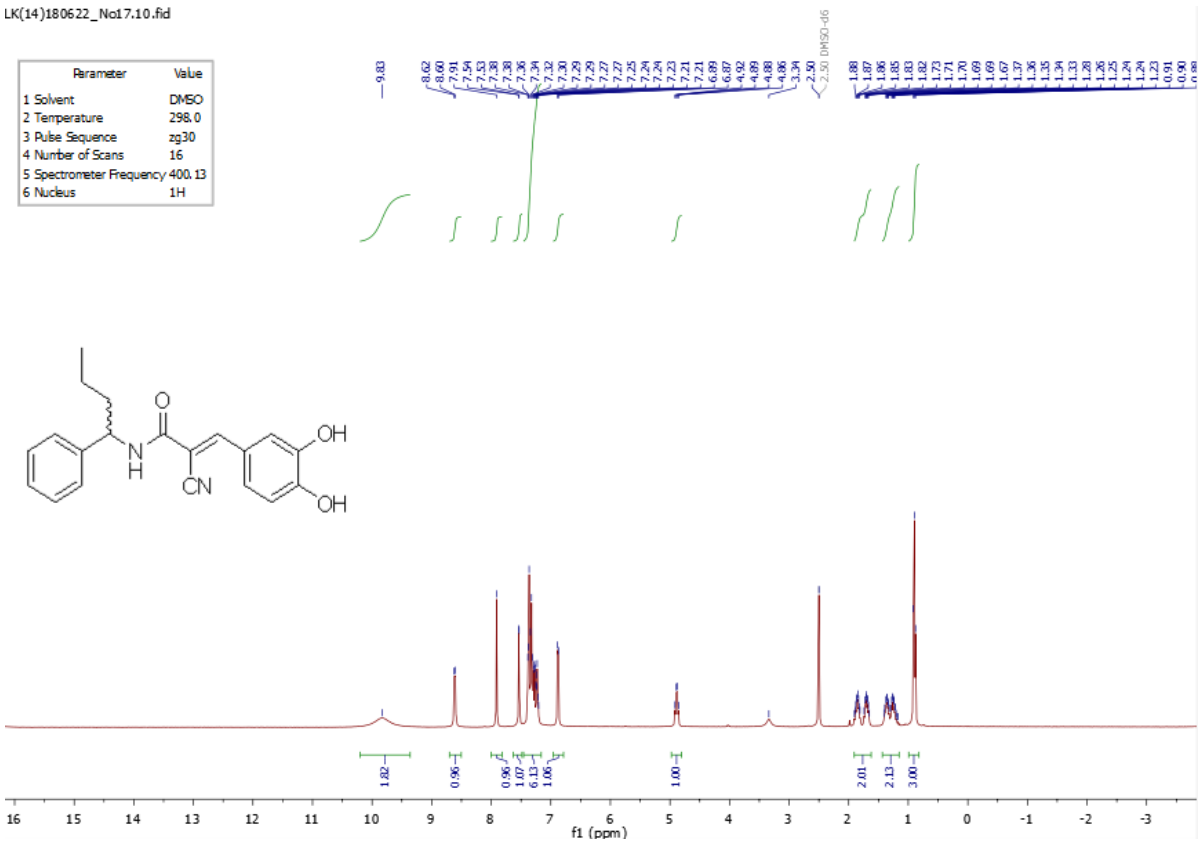


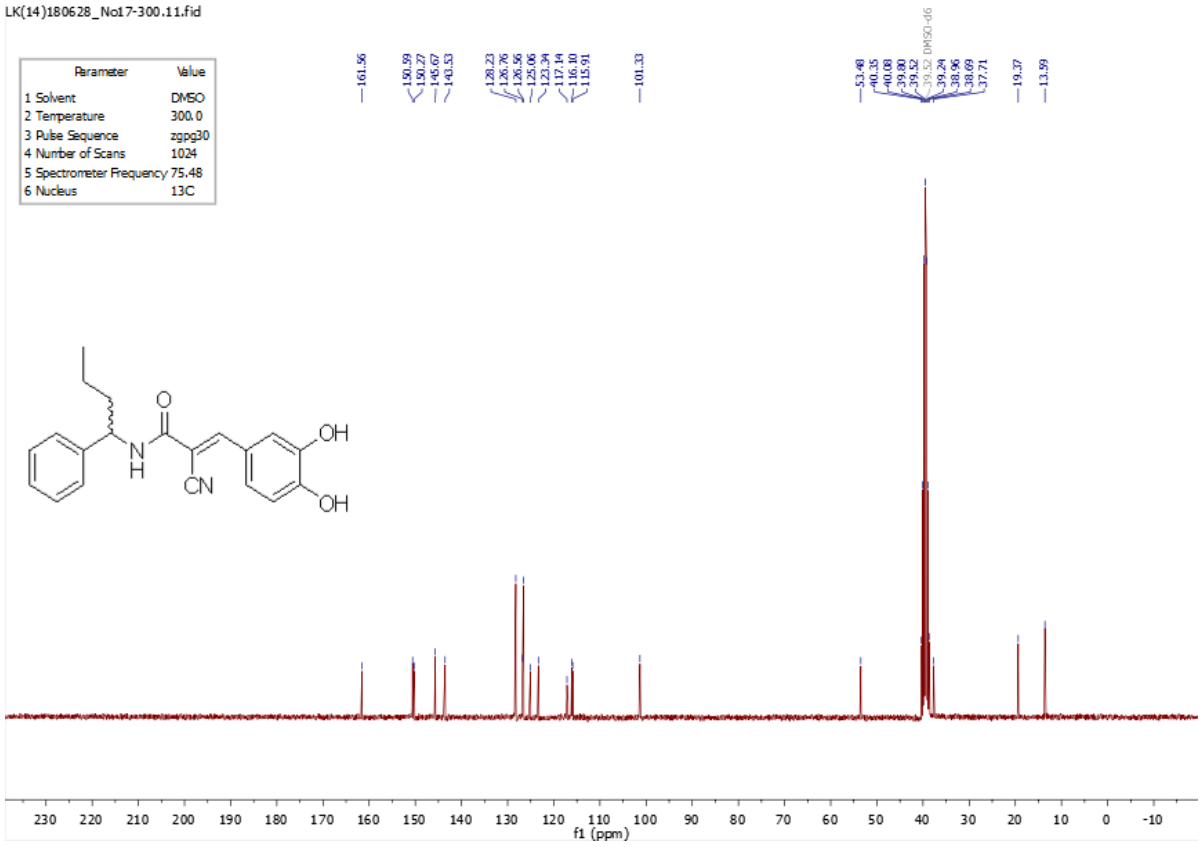
(*E*)-2-Cyano-3-(4-iodophenyl)-*N*-(1-phenylbutyl)acrylamide (10)



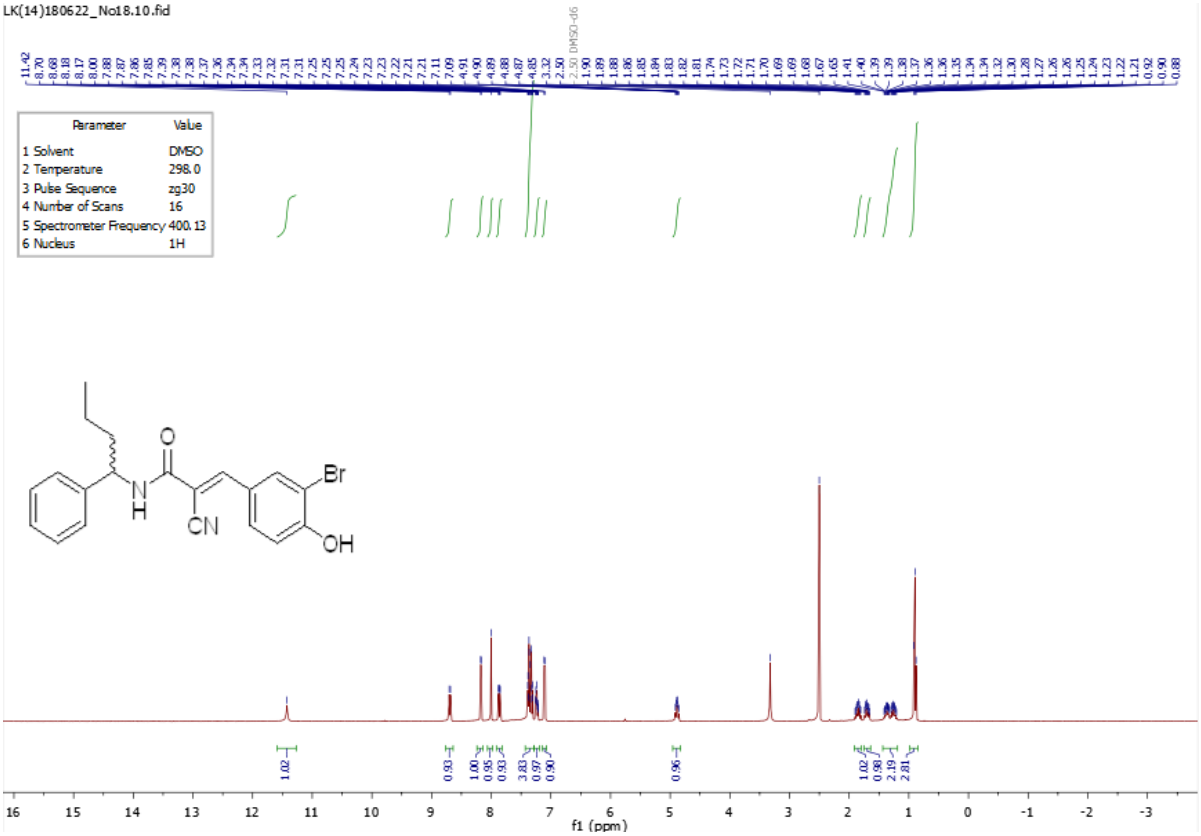


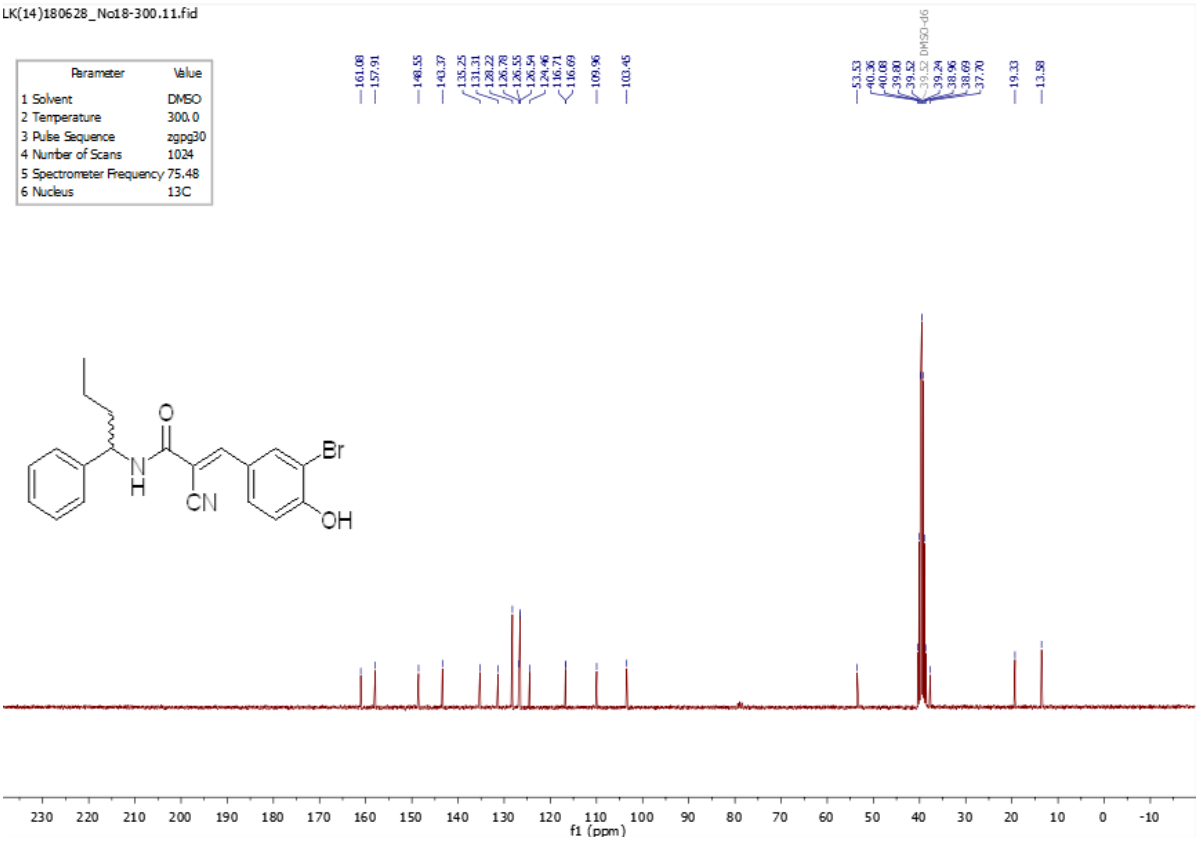
(*E*)-2-Cyano-3-(3,4-dihydroxyphenyl)-*N*-(1-phenylbutyl)acrylamide (11)



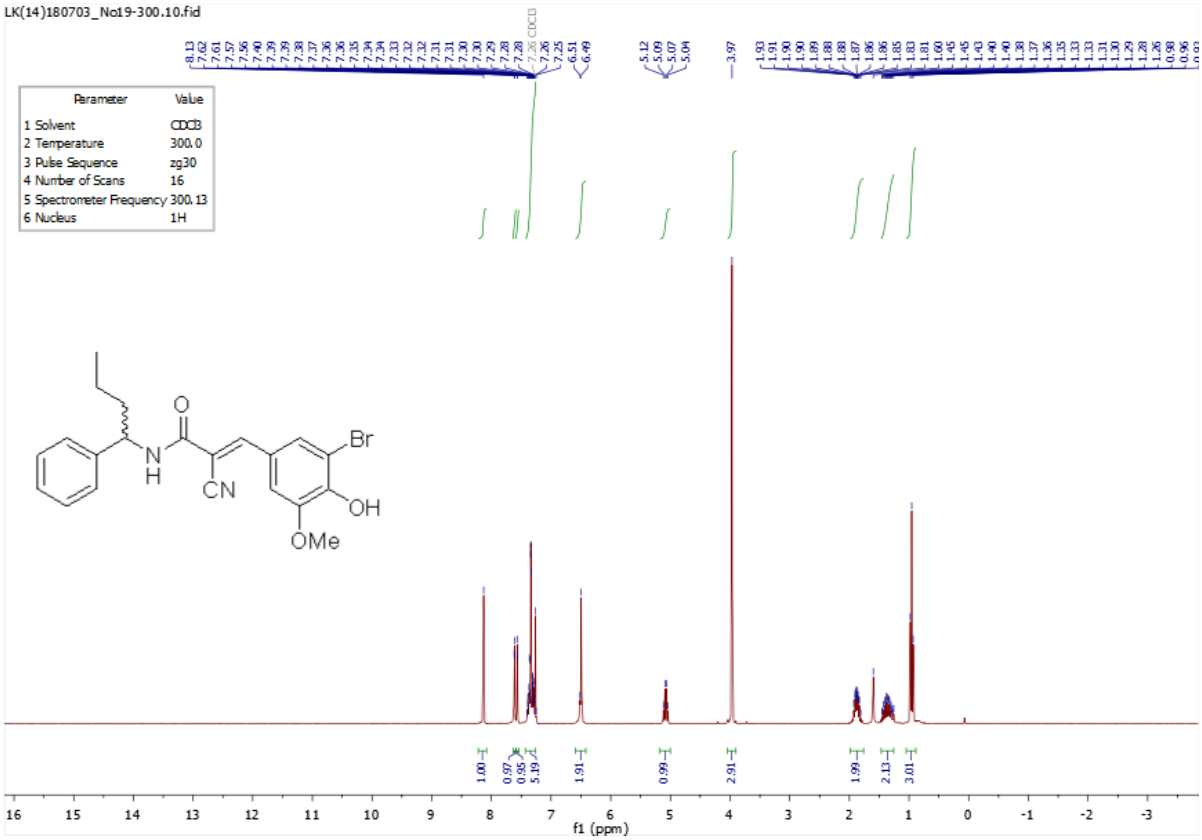


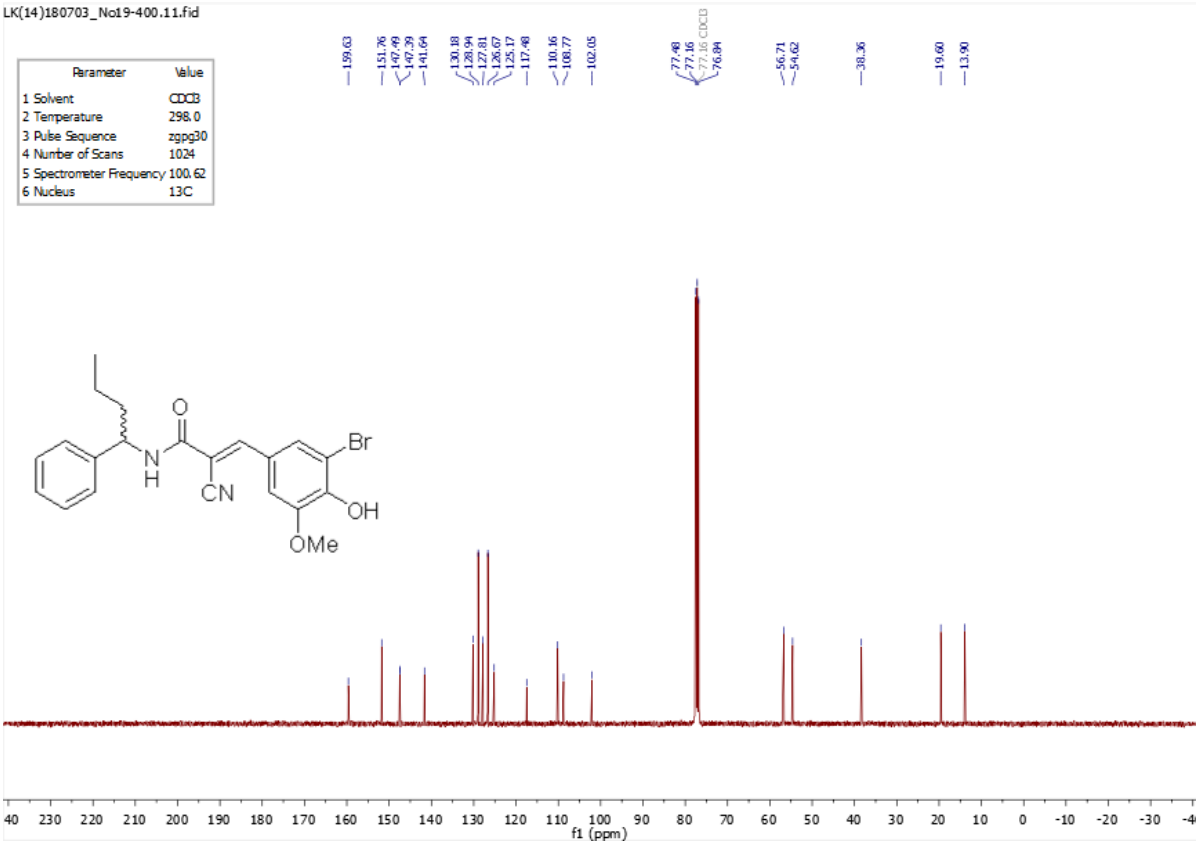
(*E*)-3-(3-Bromo-4-hydroxyphenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (12)



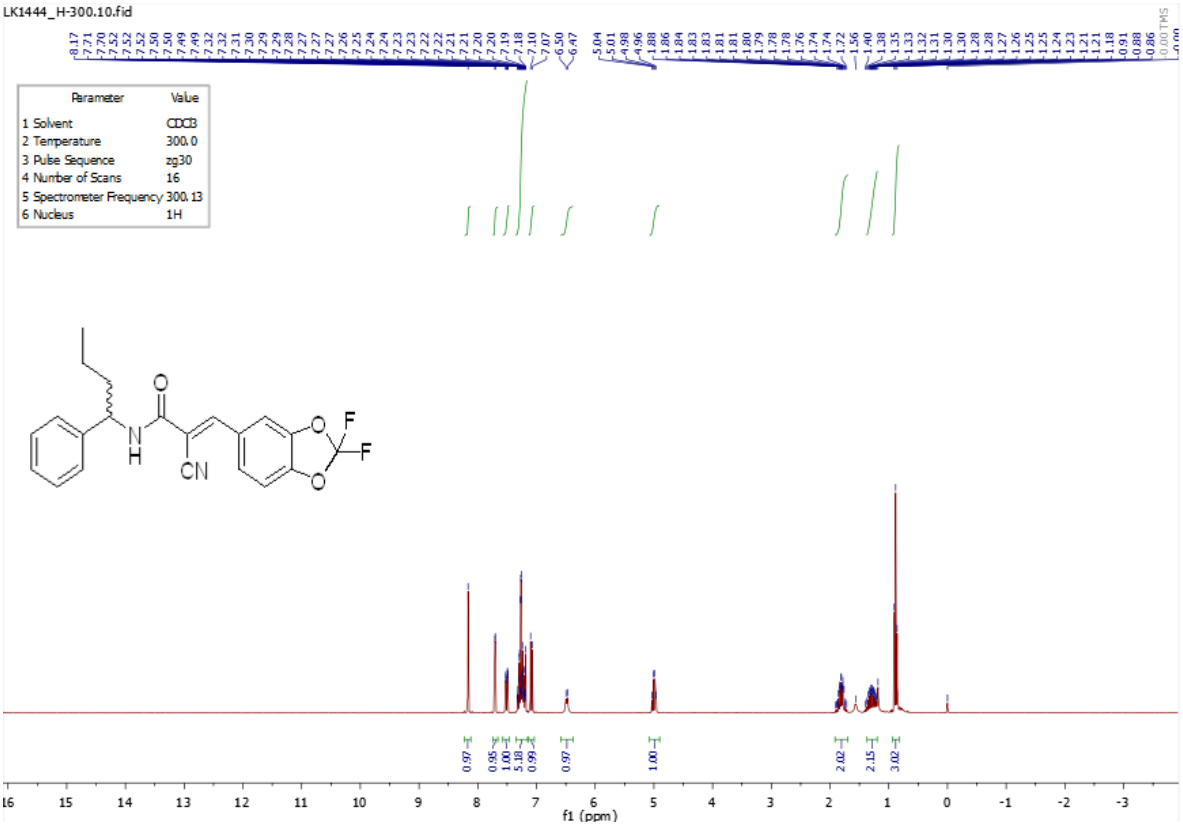


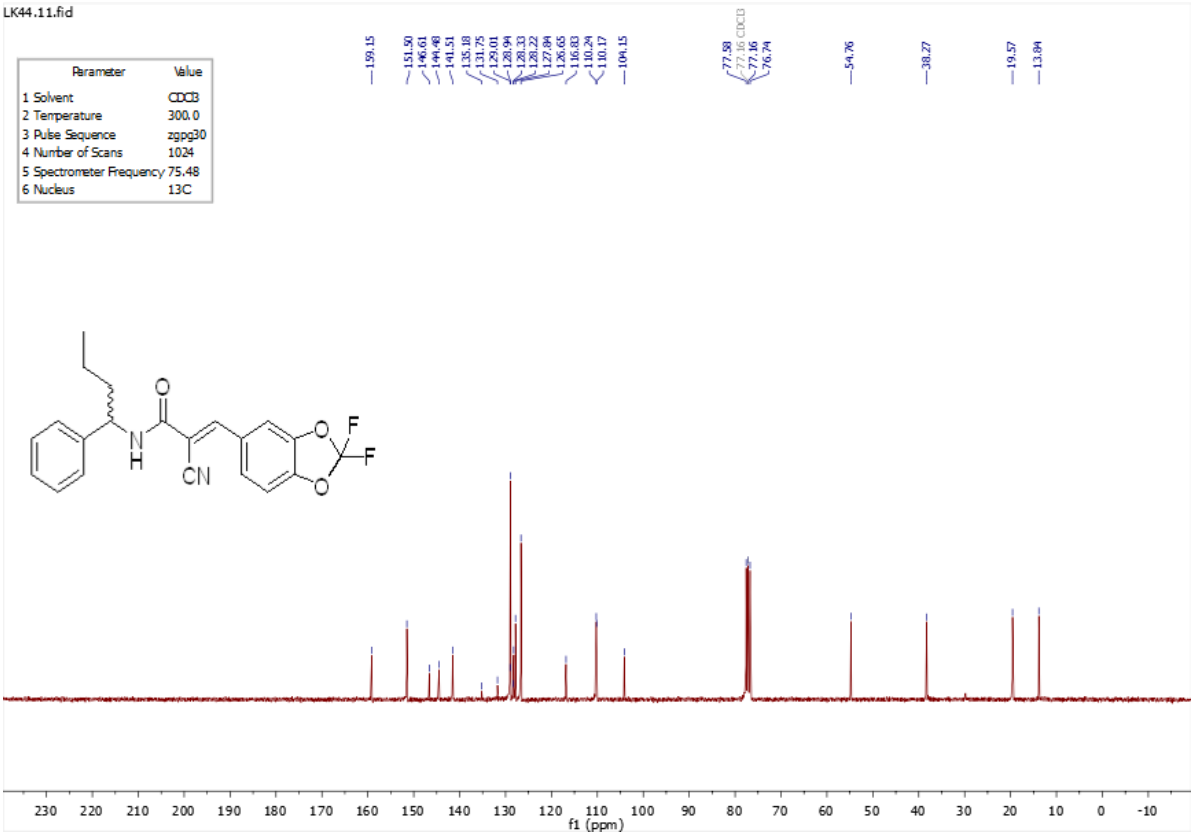
(*E*)-3-(3-Bromo-4-hydroxy-5-methoxyphenyl)-2-cyano-*N*-(1-phenylbutyl)acrylamide (13)



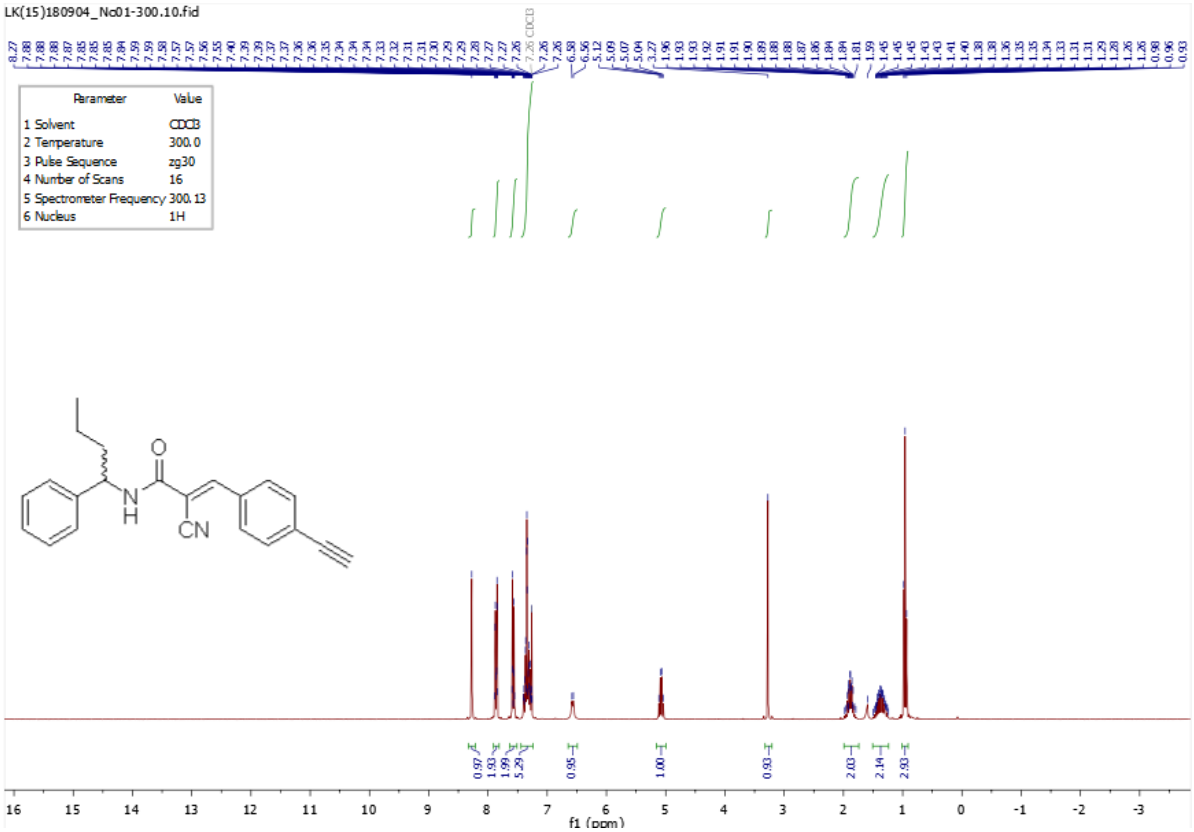


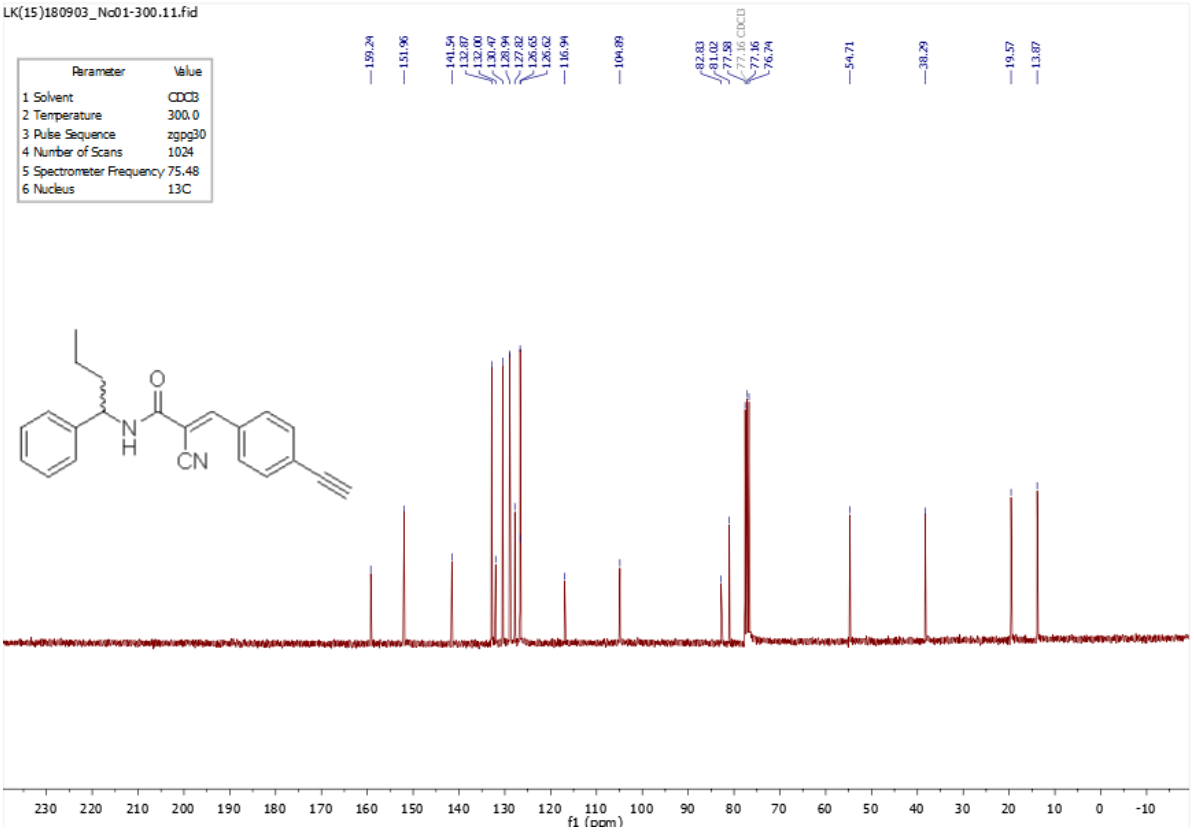
(*E*)-2-Cyano-3-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)-*N*-(1-phenylbutyl)acrylamide (14)



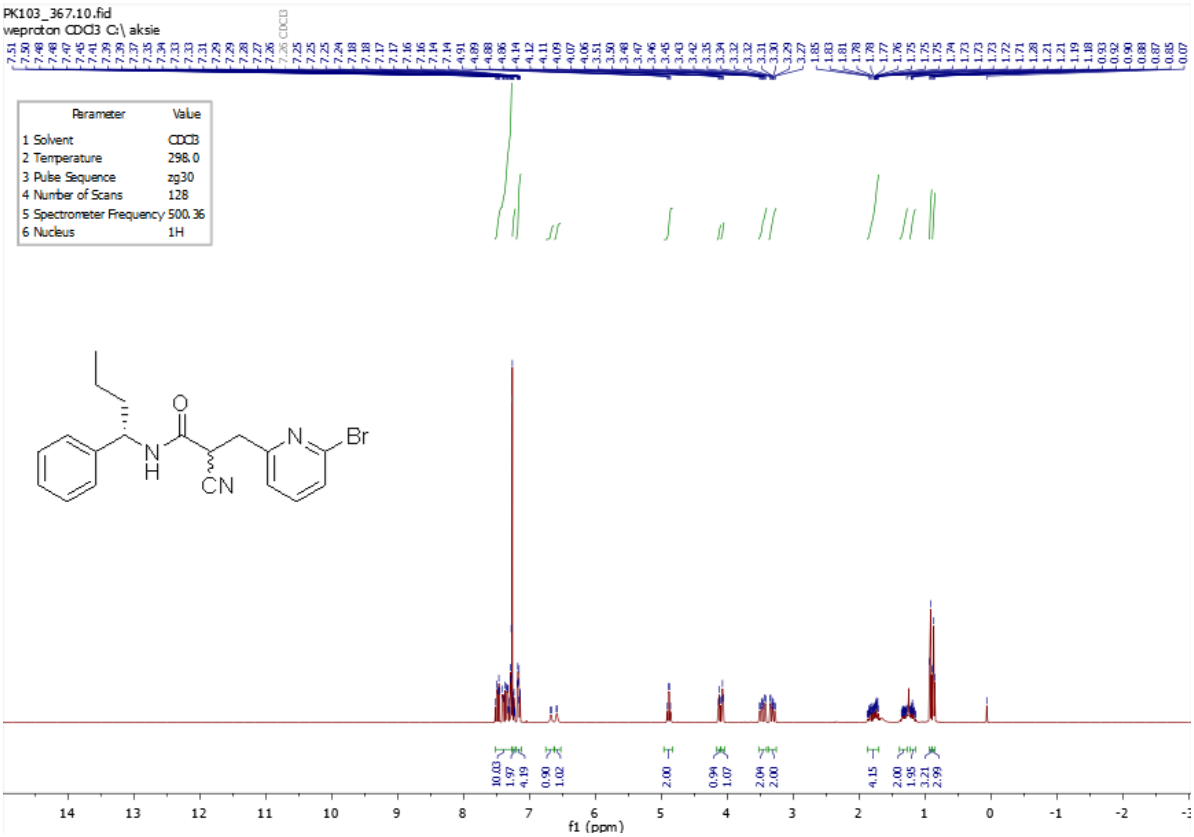


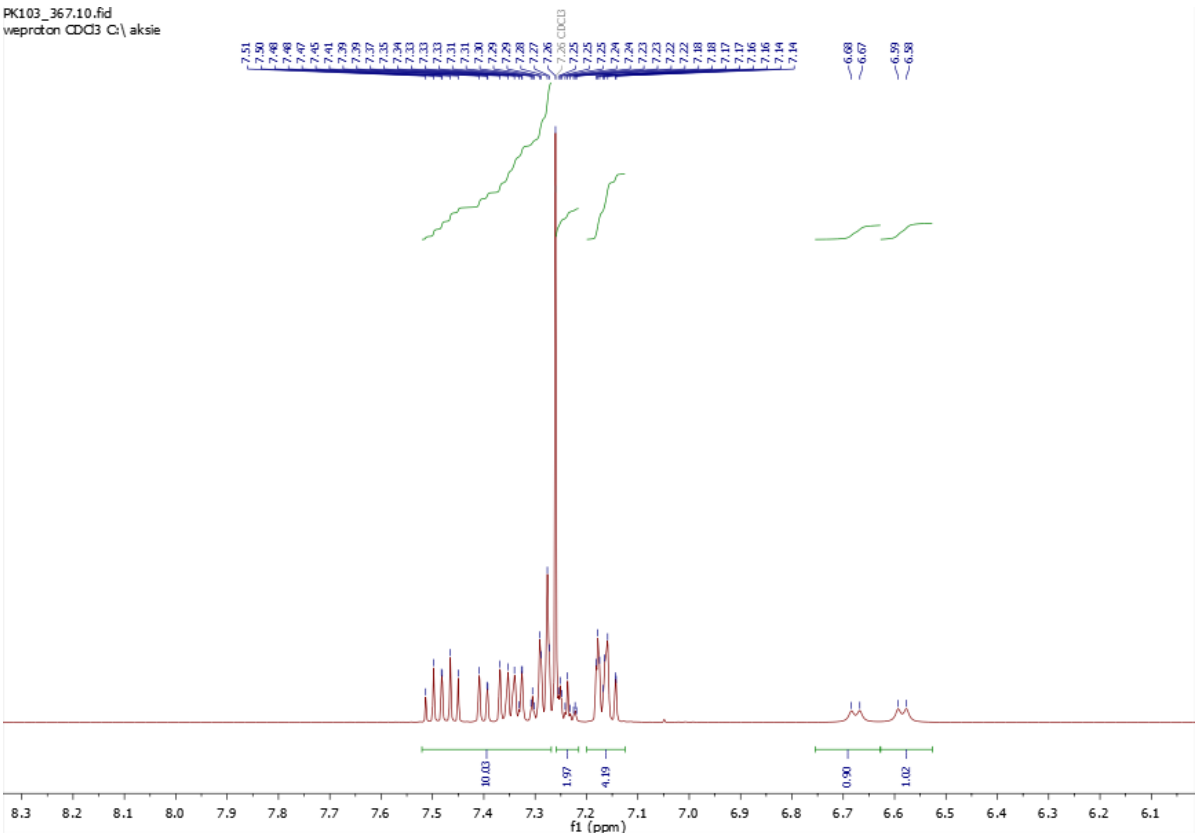
(*E*)-2-Cyano-3-(4-ethynylphenyl)-*N*-(1-phenylbutyl)acrylamide (15)

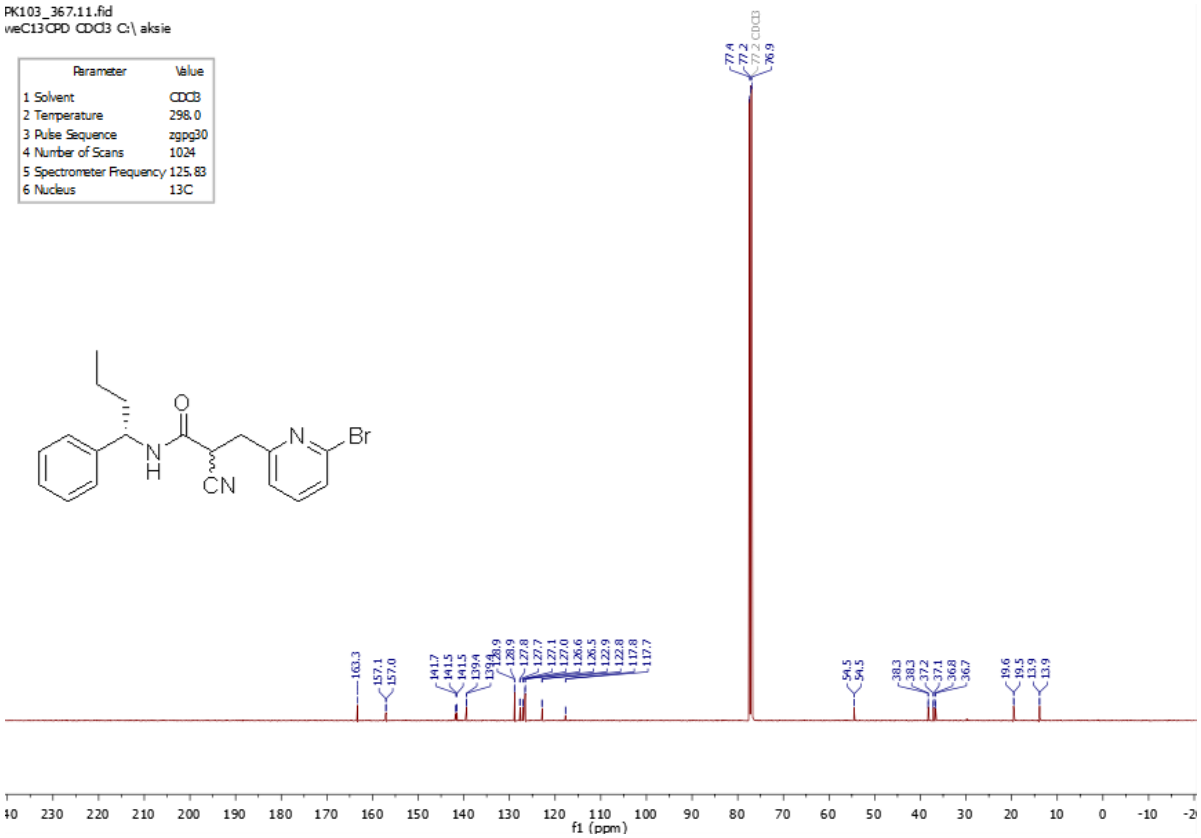




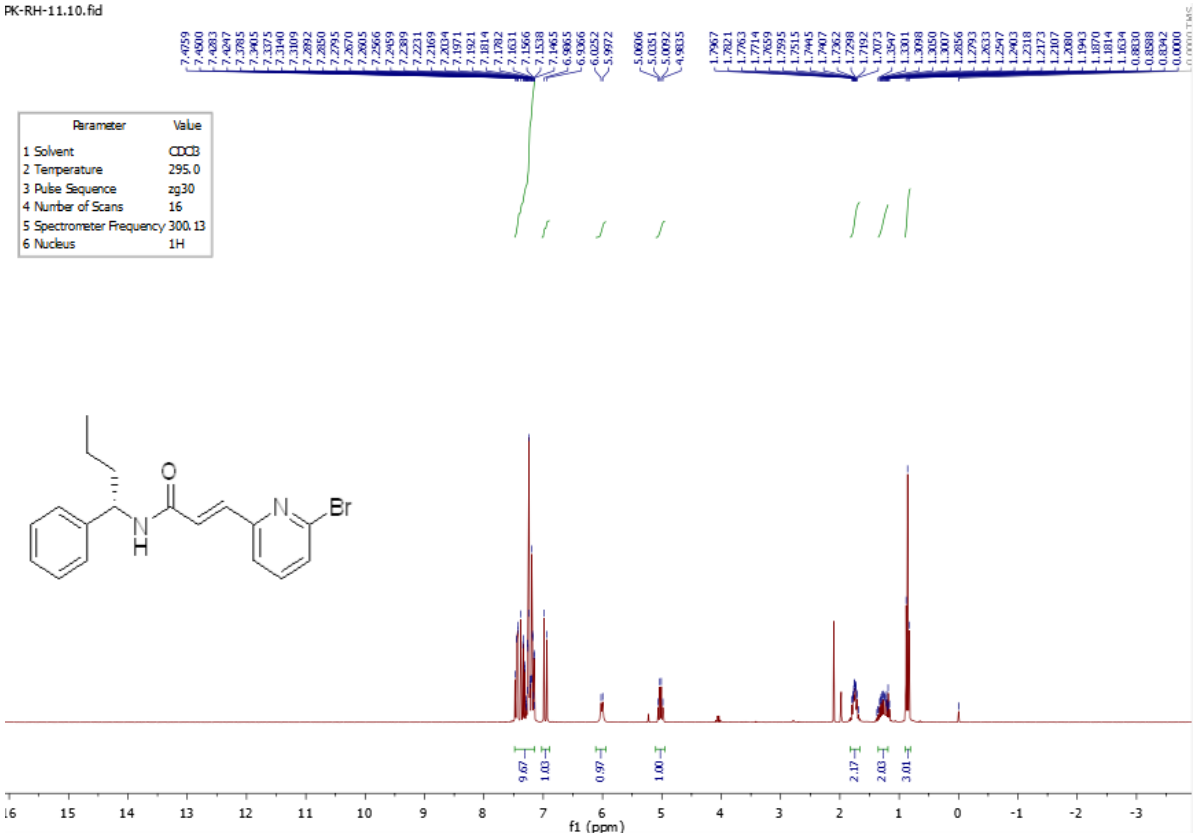
3-(6-Bromopyridin-2-yl)-2-cyano-*N*-((*S*)-1-phenylbutyl)propanamide (16)

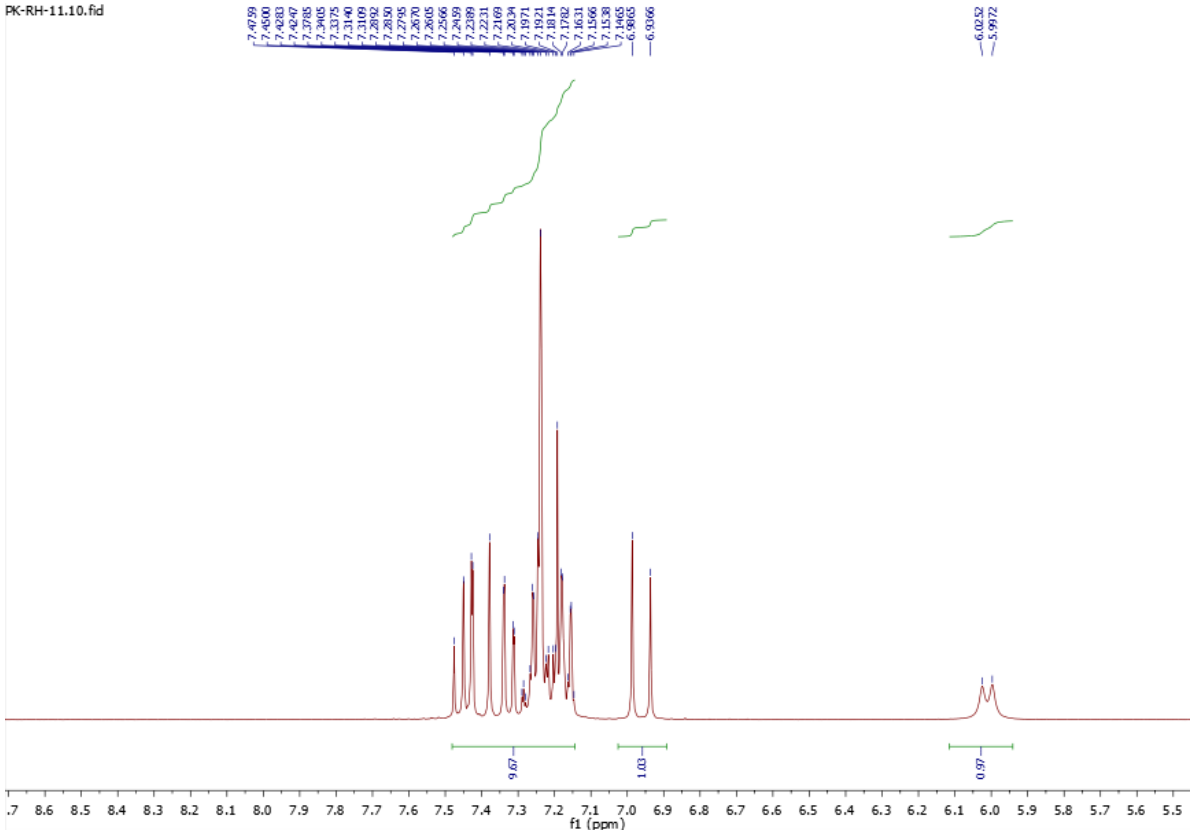


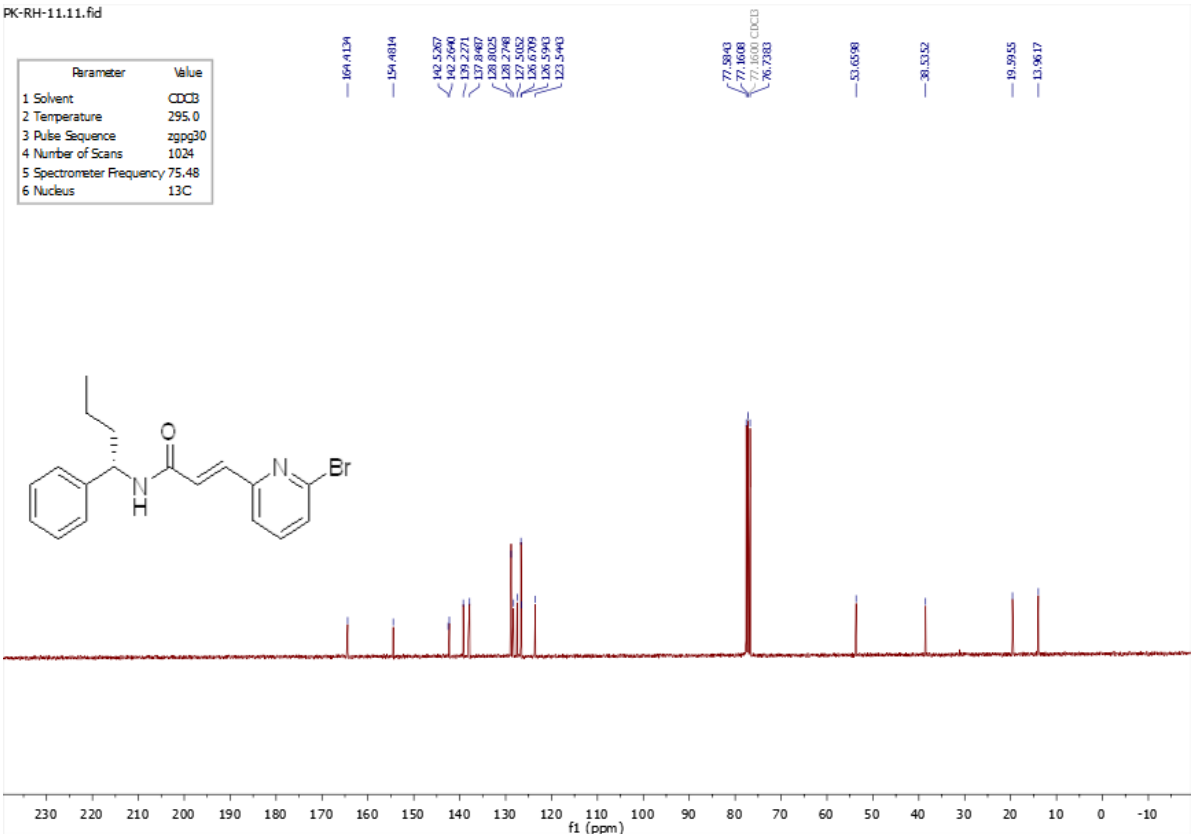



PK103_367.11.fid
weC13CPD CDCl3 C:\ aksie
Parameter Value
1 Solvent CDCl3
2 Temperature 298.0
3 Pulse Sequence zgpg30
4 Number of Scans 1024
5 Spectrometer Frequency 125.83
6 Nucleus 13C
O
N
H
CN
N
Br
77.4
77.2
CDCl3
76.9
163.3
157.1
157.0
141.7
141.5
141.5
139.4
128.9
127.8
127.7
127.1
127.0
126.6
126.5
122.9
122.8
117.8
117.7
54.5
54.5
38.3
38.3
37.2
37.1
36.8
36.7
19.6
19.5
13.9
13.9
f1 (ppm)

(*S*,*E*)-3-(6-Bromopyridin-2-yl)-*N*-(1-phenylbutyl)acrylamide (17)






PK-RH-11.11.fid
Parameter Value
1 Solvent CDCl3
2 Temperature 295.0
3 Pulse Sequence zgpg30
4 Number of Scans 1024
5 Spectrometer Frequency 75.48
6 Nucleus 13C
169.4134
154.4814
142.5267
142.2690
139.2271
137.8487
128.8025
128.2798
127.5052
126.6709
126.5943
123.5443
77.5940
77.1608
77.1600 CDCl3
76.7383
53.6598
38.5352
19.5955
13.9617
O
N
H
N
Br
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)