Supplementary Information

Proteome-wide screening for probe development in activity-based protein profiling

Youlong Fan[1-3,7], Yaping Peng[4,7], Jixiang He[5,7], Jun Xiong[6,7], Kang Duan[1], Keke Liu[5], Caiping Tian[5], Ling Fu[5], Hao Chi[4]*, Zhengqiu Li[1]*, Jing Yang[2,3,5]*

[1] College of Pharmacy, Jinan University, Guangzhou, China

[2] Guangzhou National Laboratory, Guangzhou International Bio Island, Guangzhou, China

[3] Guangzhou Medical University, Guangzhou, China

[4] Key Laboratory of Intelligent Information Processing of Chinese Academy of Sciences (CAS); Institute of Computing Technology, CAS; University of Chinese Academy of Sciences, Beijing, China

[5] State Key Laboratory of Proteomics, National Center for Protein Sciences • Beijing, Beijing, China

[6] School of Pharmacy, Xianning Medical College, Hubei University of Science and Technology, Hubei, China

[7] These authors contribute equally to this work.

*Correspondence should be addressed to H.C. (Email: chihao@ict.ac.cn ORCID: 0000-0002-7898-2599), Z.L (Email: pharmlzq@jnu.edu.cn ORCID: 0000-0002-0433-2147) or J.Y. (Email: yang_jing@gzlab.ac.cn ORCID: 0000-0001-8486-273X)

Supplementary Information

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Supplementary Note 1: Tailoring the chemoproteomic workflow. In theory, omitting enrichment could reduce sample processing time and labor, but also compromise the profiling depth of probe-labeled products (**Supplementary Fig. 1a**). Nevertheless, we first tested whether the original pChem platform retained sufficient sensitivity and accuracy to identify probe-derived modifications (PDMs) and their localized sites from MS datasets generated without enrichment. Initially, we chose an alkyne probe called IPM that contains the classic iodoacetamide warhead for cysteine bioconjugation (**Supplementary Fig. 1b**). HEK293T cell lysates were labeled with IPM at different concentrations, and then processed to generate isotopically tagged peptides (without enrichment). As such, the whole process could be completed within one day, while reducing the required starting material by an order of magnitude. Then, the resulting peptides were analyzed by LC-MS/MS, followed by automated analysis with pChem (**Supplementary Data 1**). Surprisingly, pChem reliably identified the targeted conjugation product (Δ252.122 Da) on cysteine residues (localization probability 96.7 ± 1.6%) with an average mass accuracy of 2.9 ppm. This held true even for datasets from samples treated with IPM at low micromolar concentrations (**Supplementary Fig. 1c**). This performance can be attributed to the efficient open-search engine in pChem, which recovered most peptide-spectrum matches (PSMs) corresponding to the PDMs (**Supplementary Fig. 1d**). Consequently, only a few dozen such PSMs were sufficient for accurate mass calculation and precise site localization (**Supplementary Fig. 1e**). Encouraged by these results, we next analyzed non-enrichment datasets generated using other classic alkyne probes, including ENE, NPM, PPMS, VSF, NHS, and STP (**Supplementary Fig. 2a** and **Supplementary Data 1**). Similarly, pChem successfully identified the targeted PDM for each probe, consistent with our previous findings from enrichment-based datasets (**Supplementary Fig. 2b**). Collectively, these results indicate that bypassing the enrichment step does not compromise the evaluation outcomes generated by the pChem platform.

Supplementary Note 2: Benchmarking the pIon-based pipeline. We next evaluated the robustness of the pIon pipeline by varying MS parameters, which can critically affect TMT-based experiments (**Supplementary Data 2**). Using a wide precursor isolation window did not noticeably affect the outcomes regarding high-confidence PDM recognition, mass accuracy, or site localization (**Supplementary Fig. 4a**), even though it may increase co-isolation of peptides (leading to chimeric

spectra). Likewise, varying the normalized collision energies (NCEs) or using different MS instruments did not significantly alter the assessment outcomes (**Supplementary Fig. 4b, c**). Furthermore, we prepared additional IPM-labeled samples under different click chemistry conditions (**Supplementary Data 2**). Again, the assessment results described above could still be reproduced (**Supplementary Fig. 4d**). Conversely, pIon returned no significant PDM identifications when applied to negative control datasets (e.g., probe-free samples treated with DMP-tag, or probe-labeled samples without DMP-tag conjugation). Together, these results demonstrate the robustness of the pIon-enabled pipeline.

Furthermore, we applied pIon to analyze datasets generated using the additional classic alkyne probes mentioned above. As expected, pIon reproduced the assessment results obtained from previous pChem analyses of enrichment MS data. All targeted PDMs were successfully identified and localized to the expected residues (**Supplementary Fig. 5a** and **Supplementary Data 2**). Notably, this was achieved in a more cost-efficient manner without compromising mass accuracy or site localization precision. We also compared pIon with the state-of-the-art FragPipe platform by searching the same non-enrichment datasets. FragPipe required manual inspection of output mass shifts, whereas pIon automated this process. Although FragPipe achieved sub-ppm mass accuracy (median deviation between calculated and theoretical PDM masses: 0.74 ppm; range: 0.3–1.3 ppm), pIon performed even better, with a median deviation of 0.46 ppm (**Supplementary Fig. 5b**). This high accuracy facilitates the assignment of *bona fide* molecular formulas.

Supplementary Note 3: Re-evaluation of diverse ABPP probes. Over the past decade, numerous residue-specific bioconjugation chemistries targeting Cys[1-3], Lys[4], Tyr[5], His[6,7], and Asp/Glu[8,9] have been developed. However, their reactivity and selectivity have not been compared in an unbiased, head-to-head manner. Therefore, we used pIon to re-evaluate a selected group of recently reported methods that target different amino acids via electrophilic, nucleophilic, or redox mechanisms. To this end, we first synthesized 19 alkyne probes based on previously reported bioconjugation warheads (**Supplementary Fig. 6a**). 293T cell lysates were treated with each probe at 1 mM, conjugated to a DMP-tag via click chemistry, processed into tryptic peptides, and analyzed by LC-MS/MS. The resulting data were subjected to pIon analysis with IPM and NHS as references (**Supplementary Data 2**). As a result, most residue-specific warheads exhibited relatively weak overall reactivity toward their intended

target residues (**Supplementary Fig. 6b**), precluding their immediate use in ABPP. This characteristic, however, may offer opportunities for repurposing in targeted covalent inhibitor (TCI) design. A few warheads showed moderate reactivity. Among them, squarate (**16**)[10] and acyl imidazole (**18**)[11] served as complementary approaches for lysine profiling. ABPP analyses using **16** and **18** collectively identified 4,240 lysine sites (**Supplementary Fig. 6c** and **Supplementary Data 3**). In addition, our analysis confirmed the promiscuous reactivity (cross-reactivity) of several electrophilic warheads (**Supplementary Fig. 6d**). For example, dichlorotriazine (**12**)[12] preferentially modified lysines, with moderate to weak reactivity toward cysteines and tyrosines. Sulfonyl-fluoride (**13**)[5] preferentially modified tyrosines, with moderate reactivity toward lysines. N-acyl-N-alkyl/aryl sulfonamides (**15, 17**)[13] preferentially modified lysines, with moderate reactivity toward cysteines. Moreover, pIon revealed unexpected side reactions or off-target residue modification for 6 of the 19 tested probes (**Supplementary Fig. 6e**). For example, probe **1** bears a photocaged warhead designed to form electrophilic thionium intermediates for histidine modification under visible light. However, it was found to predominantly modify lysines. In another case, probes **4** and **5**, bearing a styrylboronic acid warhead, preferentially modified cysteines, despite literature reports that this chemistry enables metal-catalyzed conjugation to backbone amides via the Chan-Lam reaction[14].

Supplementary Note 4: Plausible mechanism of 2-pyrone-substituted alkyne-mediated bioconjugation. We proposed a reaction mechanism analogous to the Morita-Baylis-Hillman reaction[15,16] (**Supplementary Fig. 7**). The proposed mechanism initiates with the attack of a biological nucleophile (acting as a catalyst) on the pyrone ring, generating an enolate intermediate. This is plausible because an electron-withdrawing group (e.g., the alkyne) at the α-position of a Michael acceptor increases the electrophilicity of the β-carbon, facilitating nucleophilic attack[17]. The combined electron-withdrawing effects of the ketone and the electron-deficient olefin enhance the acidity of the αC–H bond. This facilitates rapid β-elimination, releasing the nucleophilic catalyst via a retro-Michael addition. Concurrently, this electronic environment stabilizes an allene intermediate formed by alkyne isomerization. Subsequent nucleophilic attack by histidine on the allene intermediate yields the final protein adducts, which can have either *Z* or *E* configuration. NMR analysis confirmed the formation of

isomeric *Z/E* products from the reaction of a non-clickable analog of **37** (lacking the terminal alkynyl group) with free histidine.

Reference:

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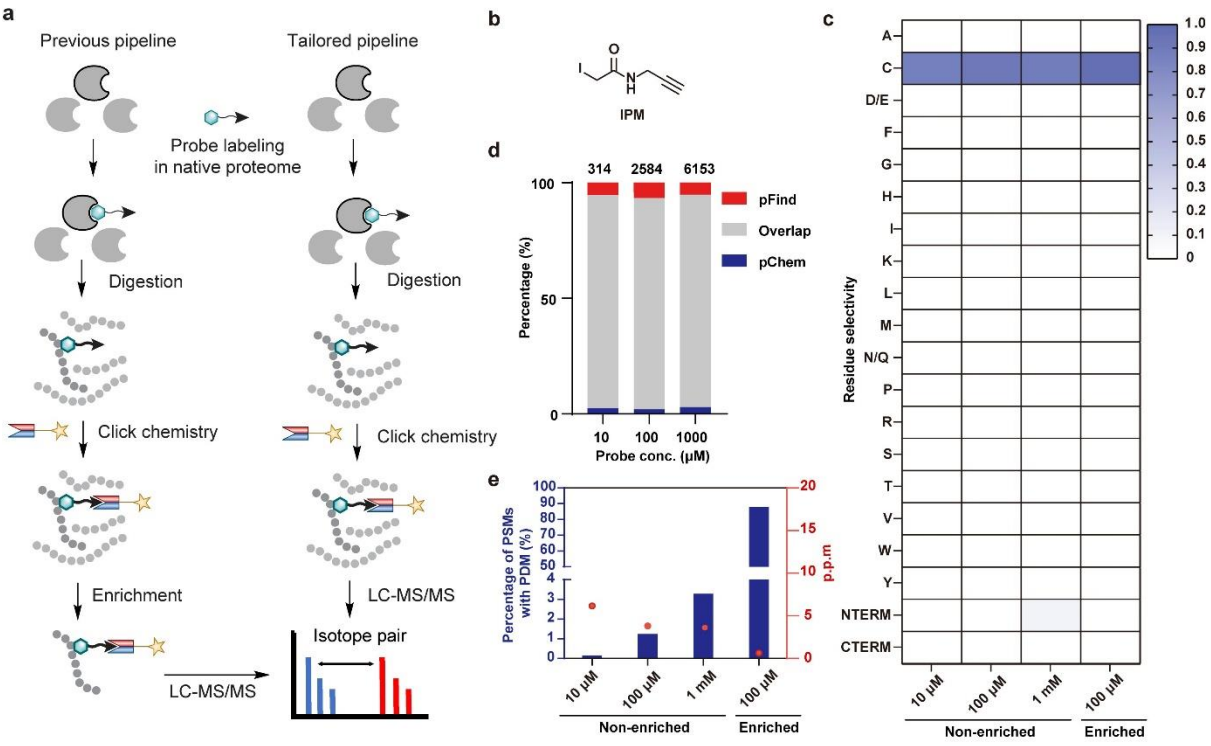
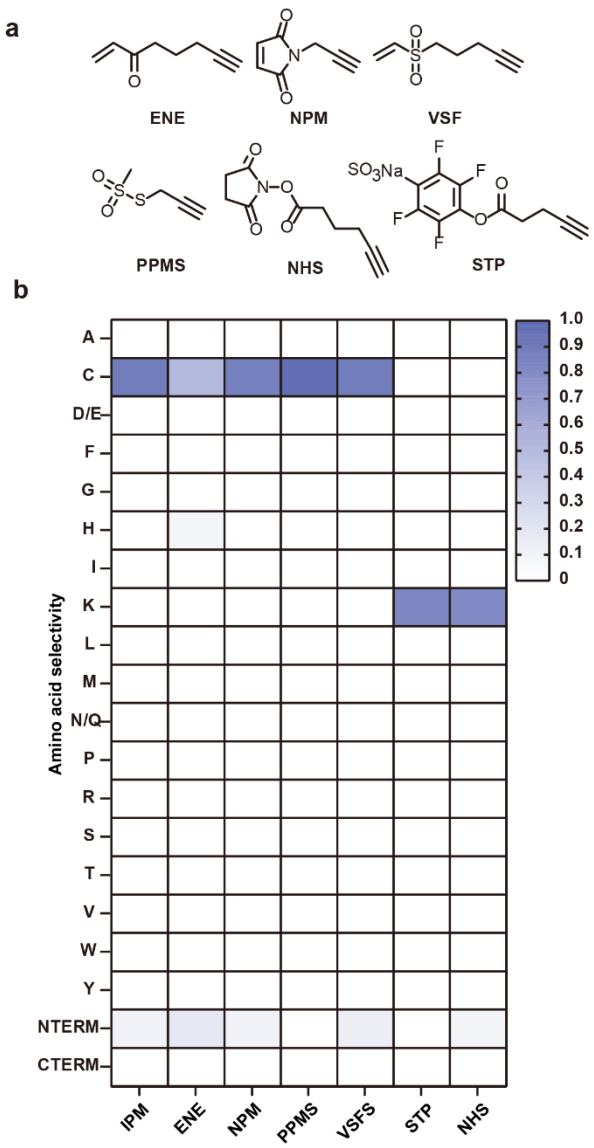
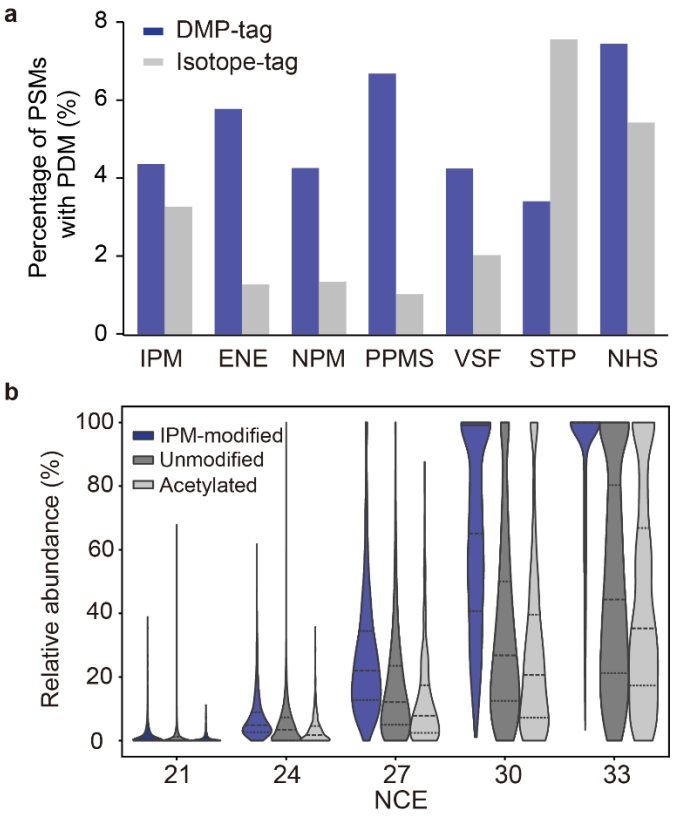


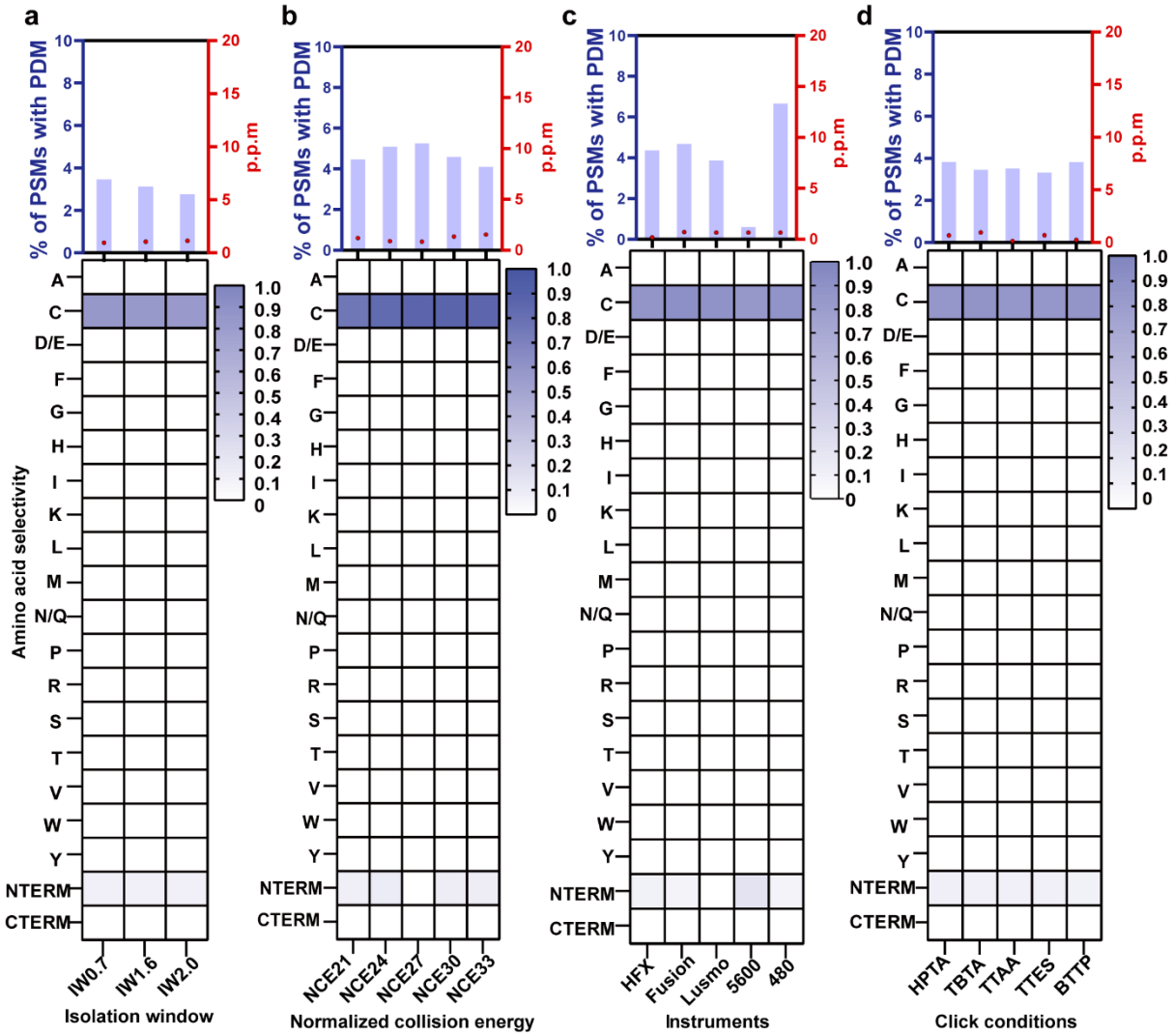
Supplementary Fig. 1. Benchmarking the tailored, pChem-based pipeline with IPM. a, Comparison of the original and the tailored (non-enrichment) pipeline for proteome-wide evaluation of covalent probes. Isotope coding of PDMs yields a paired MS1 signature that can be used to distinguish *bona fide* PDMs from endogenous and/or common modifications. **b**, Chemical structure of IPM, a classic cysteine-specific probe. **c**, Representative heatmaps from pIon analysis showing residue localization distributions for the IPM-derived modification. Datasets were generated with or without enrichment of probe-modified peptides. **d**, Stacked chart showing the overlaps of search results from pChem and pFind. **e**, Bar chart showing the proteome-wide reactivity of IPM, quantified as the percentage of PSMs assigned to the corresponding PDM. Red dots indicate the mass error (calculated vs. theoretical) for each identified PDM.



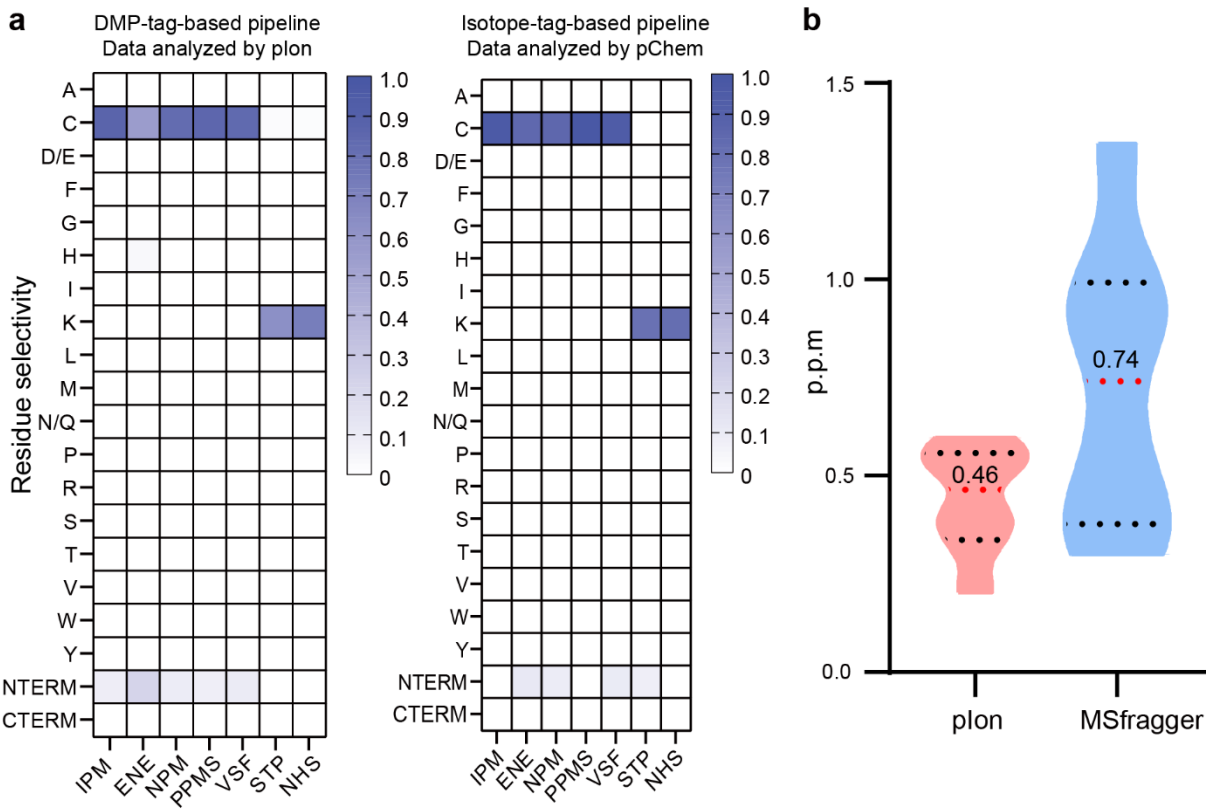
Supplementary Fig. 2. Benchmarking the tailored, pChem-based with residue-reactive probes. **a**, Chemical structures of 6 classic covalent probes with well-established residue-specific warheads. **b**, Representative heatmaps showing residue localization distributions of pChem-identified PDMs for each indicated probe.



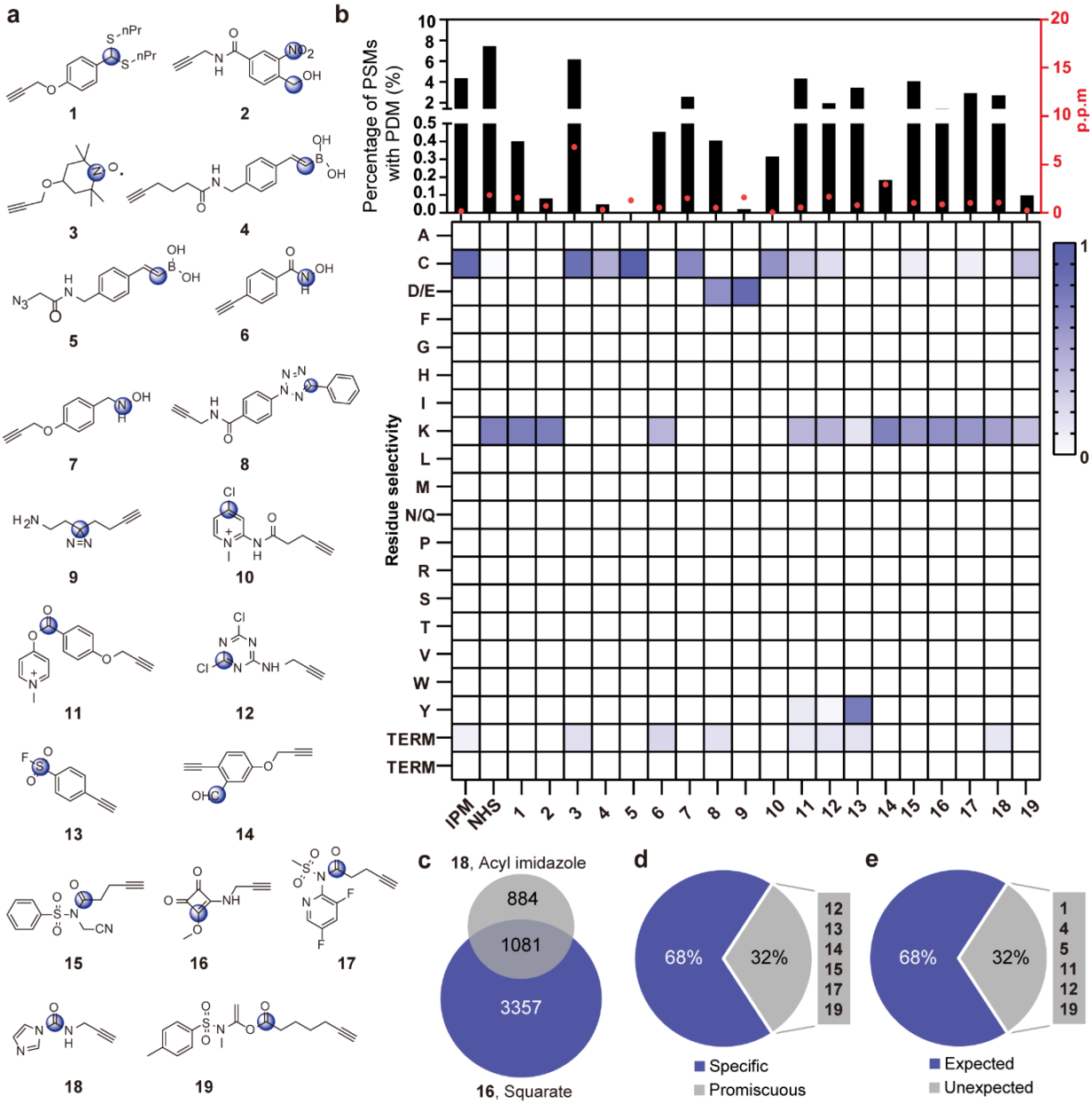
Supplementary Fig. 3. Evaluation of DMP-tag. a, Comparison of DMP-tag and isotope tags based on the percentage of PSMs assigned to each indicated PDM. **b**, Distribution of the relative abundance of the *m/z* 126.1277 reporter ion in MS/MS spectra assigned to different peptide types. Dashed lines indicate medians; dotted lines indicate the 25th and 75th percentiles.



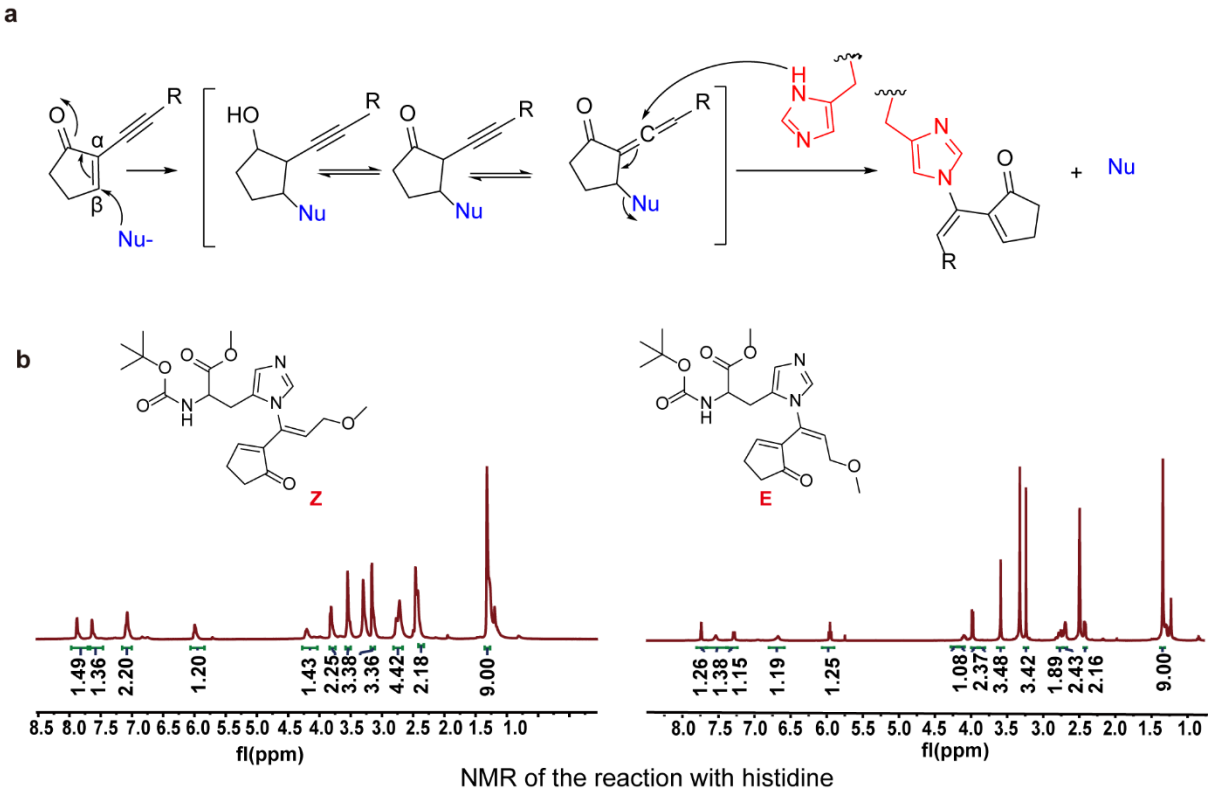
Supplementary Fig. 4. Robustness of plon. Residue localization heatmaps for the IPM-derived modification under varying conditions: (**a**) isolation window width, (**b**) normalized collision energy (NCE), (**c**) MS instrument type, (**d**) CuAAC ligand.



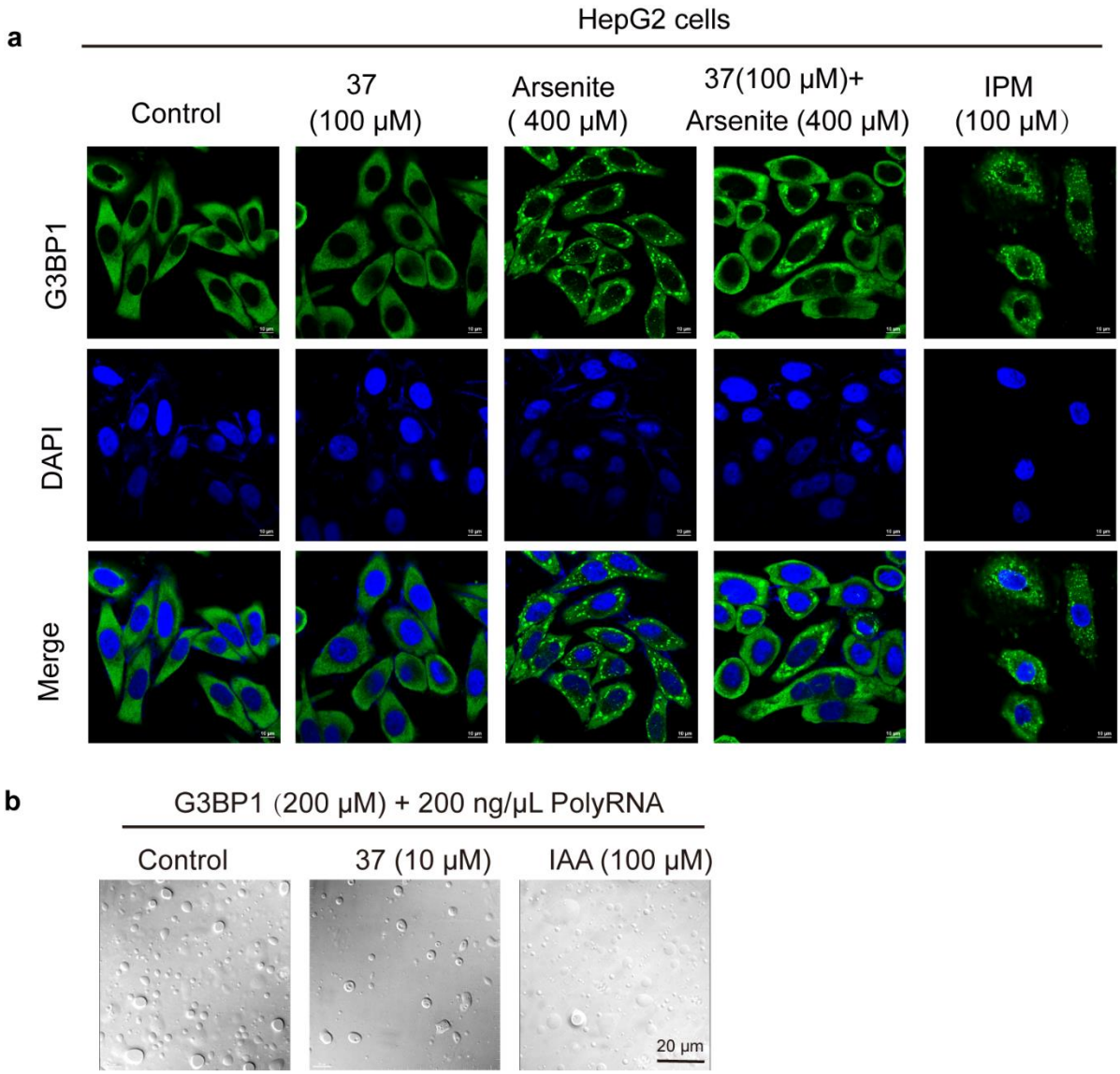
Supplementary Fig. 5. Comparison of pIon with other open search-based informatic tools. a, Residue localization heatmaps from pIon (top) and pChem (bottom) analyses for the indicated probes. **b,** Violin plots comparing mass accuracy (deviation from theoretical mass) achieved by pIon and MSFragger (via FragPipe). Red dotted lines, medians; black dotted lines, 25th and 75th percentiles.



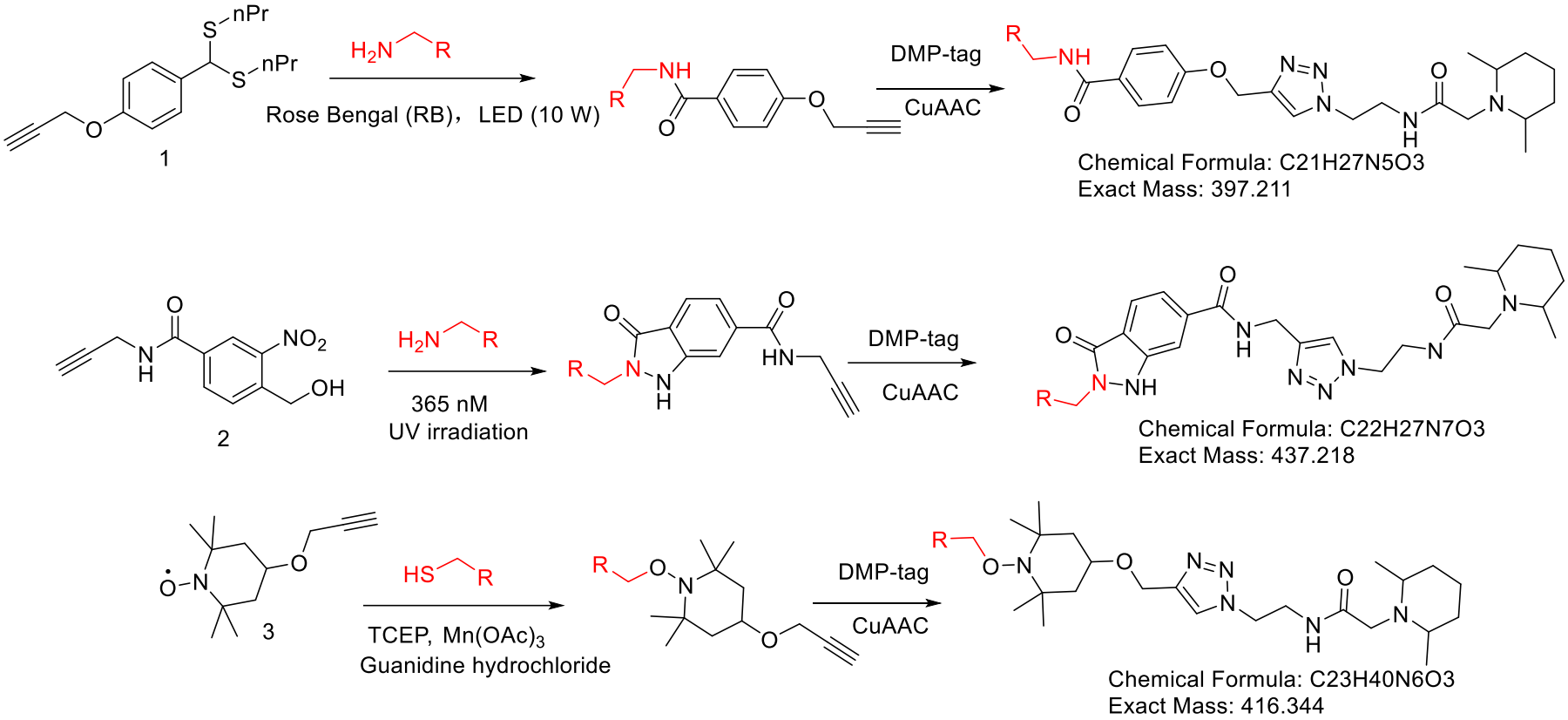
Supplementary Fig. 6. Re-assessing the proteome-wide selectivity of diverse ABPP probes. a, Chemical structures of 19 previously reported probes. **b**, Residue localization heatmaps from pIon for each probe. Bar charts showing overall reactivity (% PSMs assigned to the PDM), with red dots indicating the mass error for each probe. **c**, Venn diagram showing overlap of lysine sites identified by probes **16** and **18**. **d**, Pie chart showing proportion of probes exhibiting promiscuous residue reactivity. **e**, Pie chart showing proportion of probes for which pIon revealed unexpected side-reactions or off-target localization.



Supplementary Fig. 7. Proposed mechanism and experimental validation of histidine conjugation by the 2-pyrone-substituted alkyne warhead. a, Plausible mechanism for histidine bioconjugation by 2-pyrone-substituted alkyne. **b,** NMR analysis confirming formation of Z/E isomeric products from reaction of a non-clickable analog of **37** (lacking the terminal alkynyl group) with free histidine.



Supplementary Fig. 8. Chemical modification impairs G3BP1 phase separation and SG assembly. **a**, Fluorescence images showing effect of CEAlkyne on arsenite-induced G3BP1 puncta formation in HepG2 cells. IPM, a thiol-reactive probe, was used as a control. Scale bar, 5 μm. **b**, In vitro assay showing that CEAlkyne, but not iodoacetamide (IAA), impairs polyRNA-induced phase separation of purified G3BP1. Scale bar, 20 μm. For **a** and **b**, Images are representative of three independent experiments.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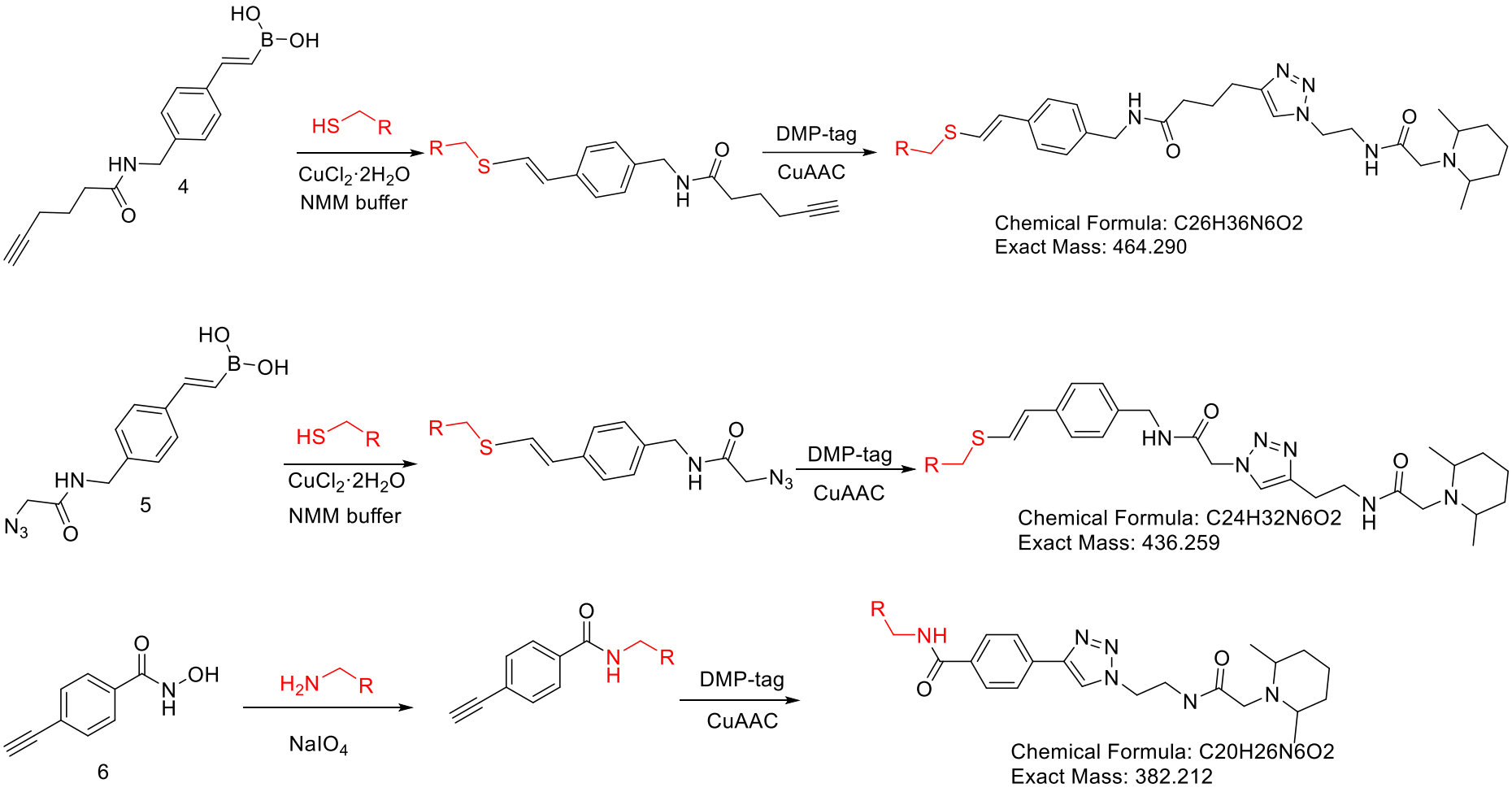
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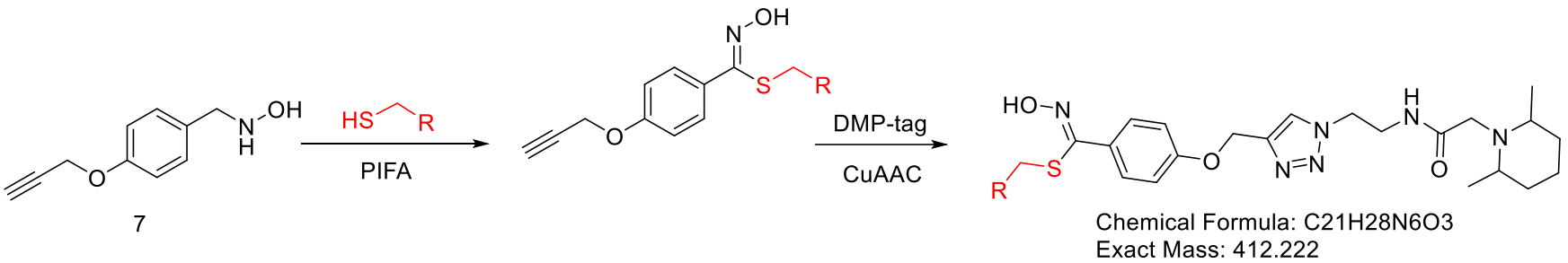
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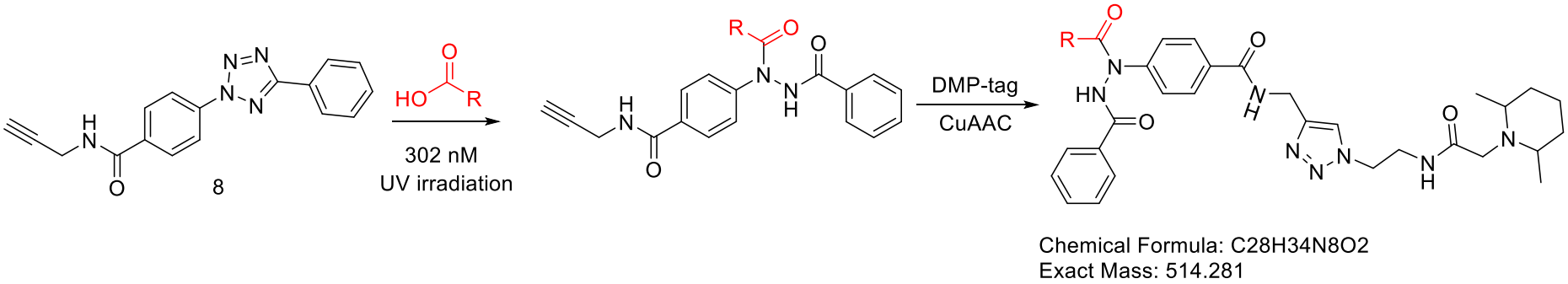
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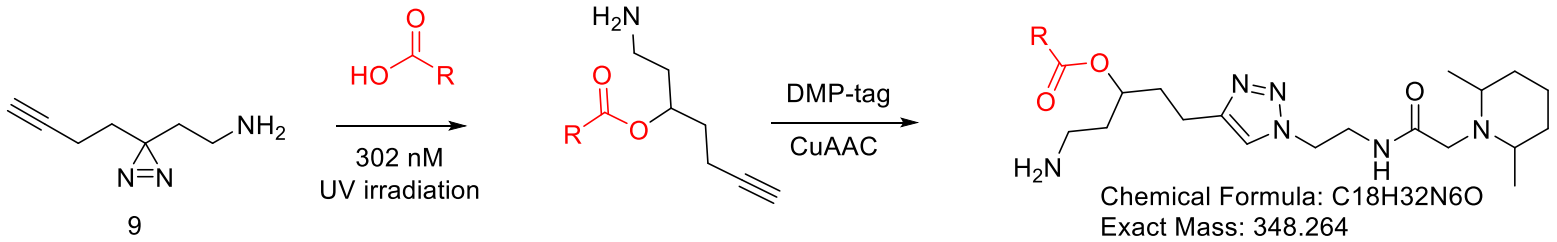
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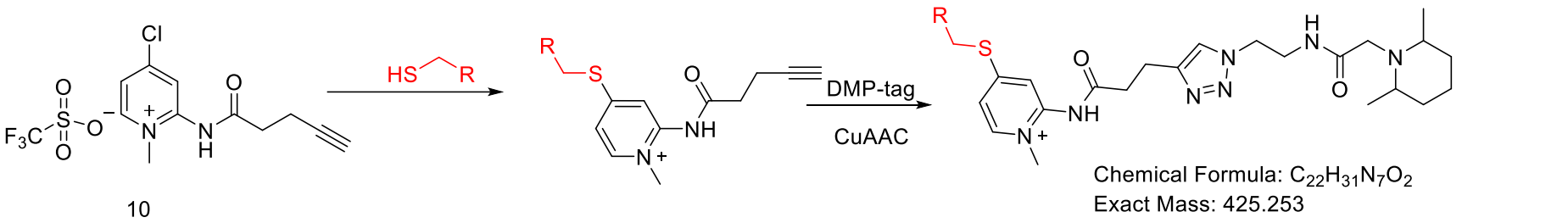
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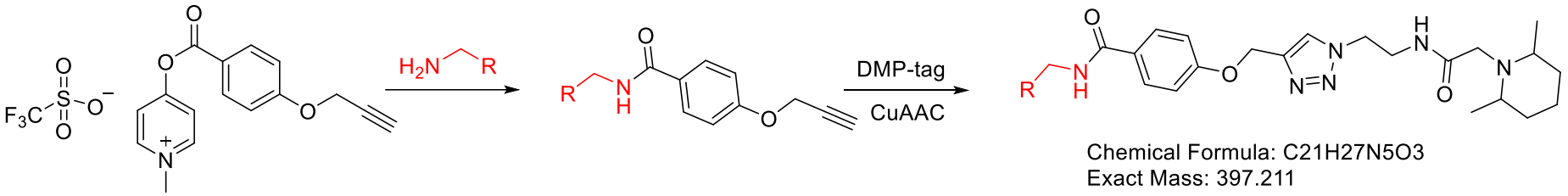
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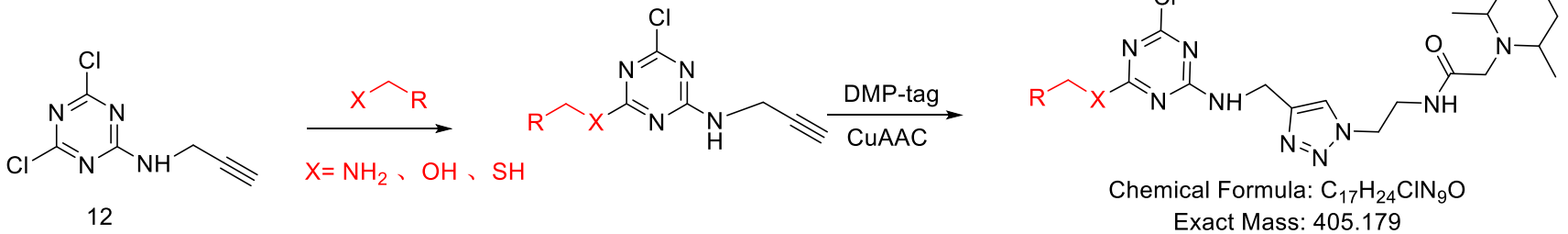
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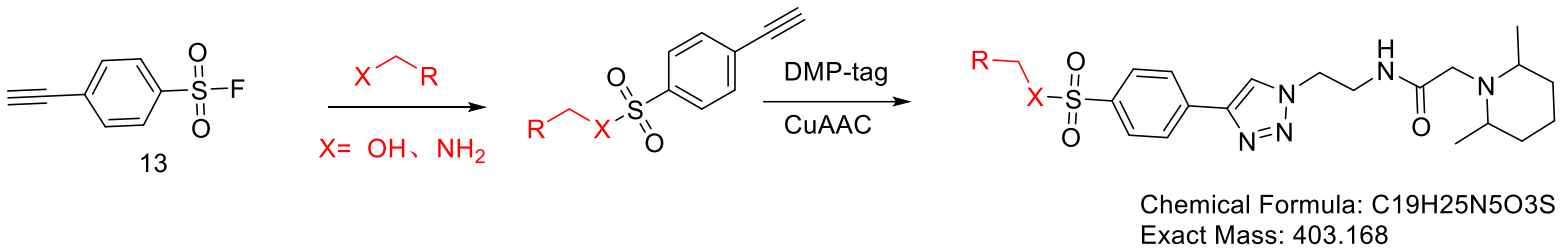
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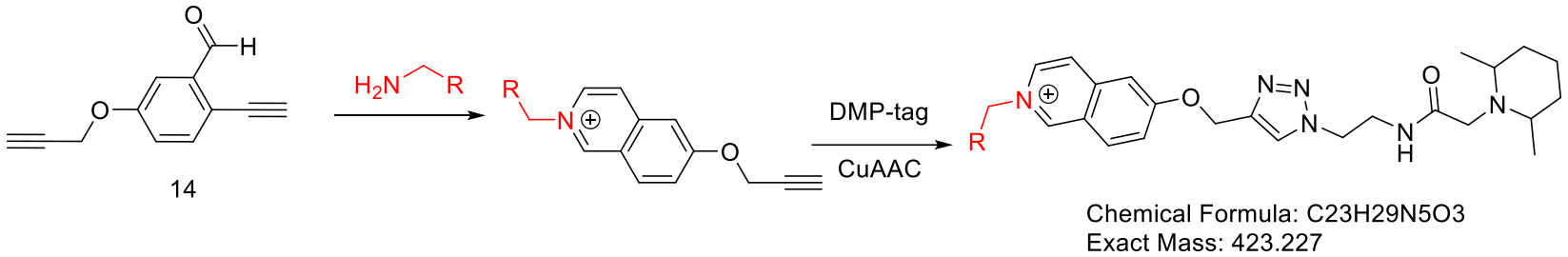
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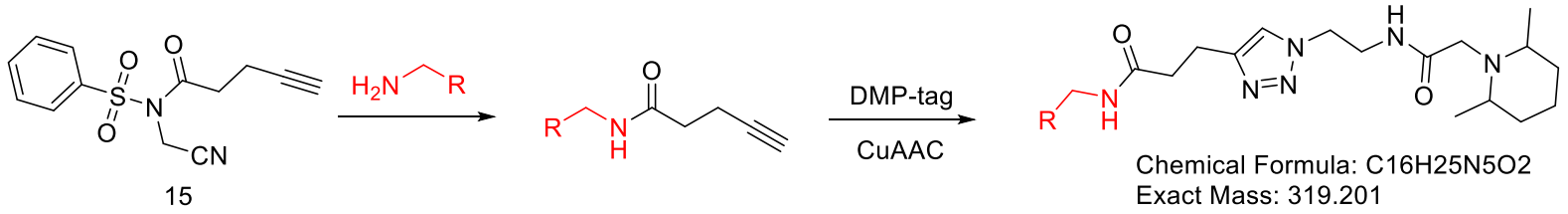
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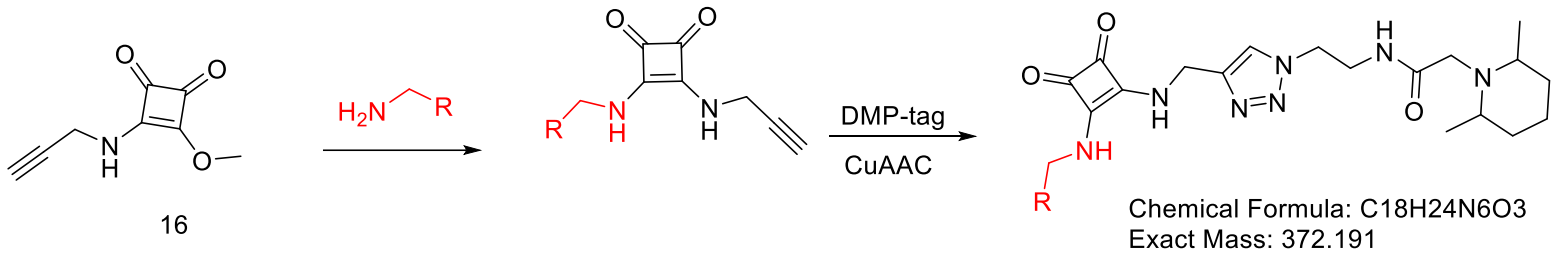
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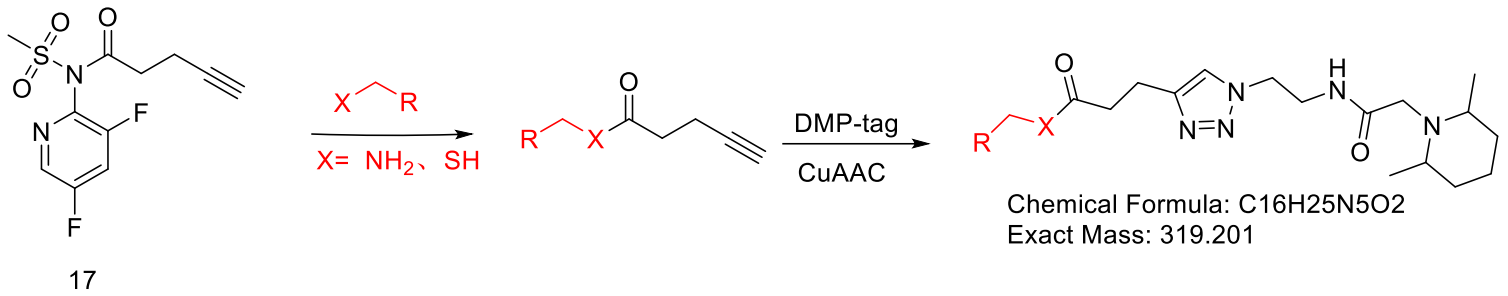
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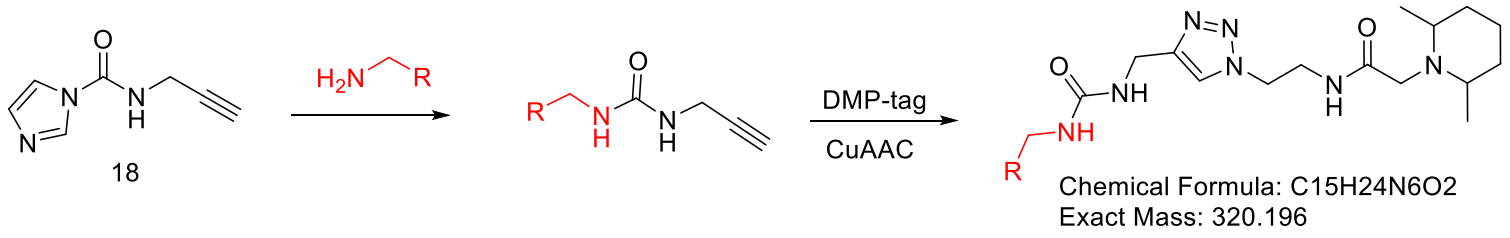
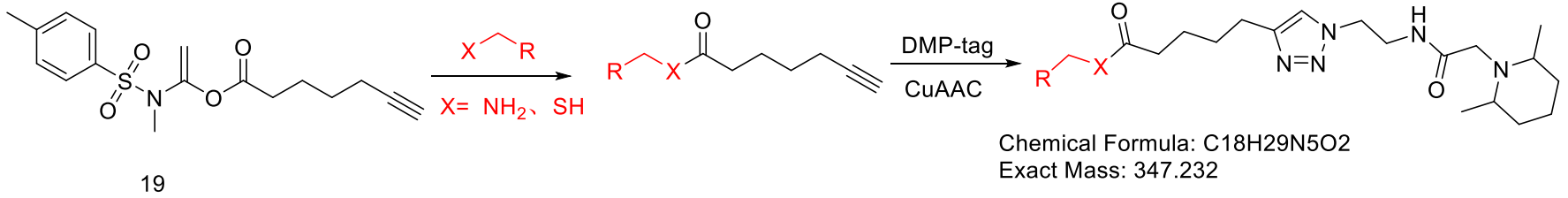
CuAAC
Chemical Formula: C16H25N5O2
Exact Mass: 319.201

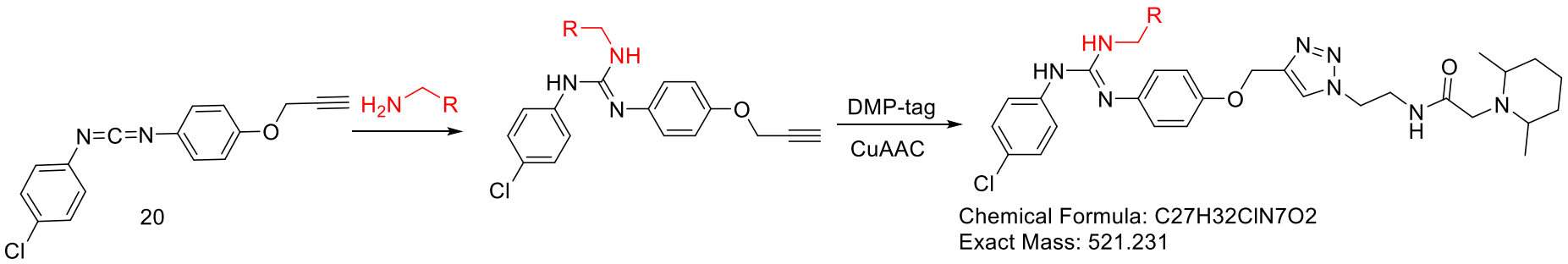
17


DMP-tag
CuAAC
Chemical Formula: C15H24N6O2
Exact Mass: 320.196

18


X= NH2、SH
DMP-tag
CuAAC
Chemical Formula: C18H29N5O2
Exact Mass: 347.232

19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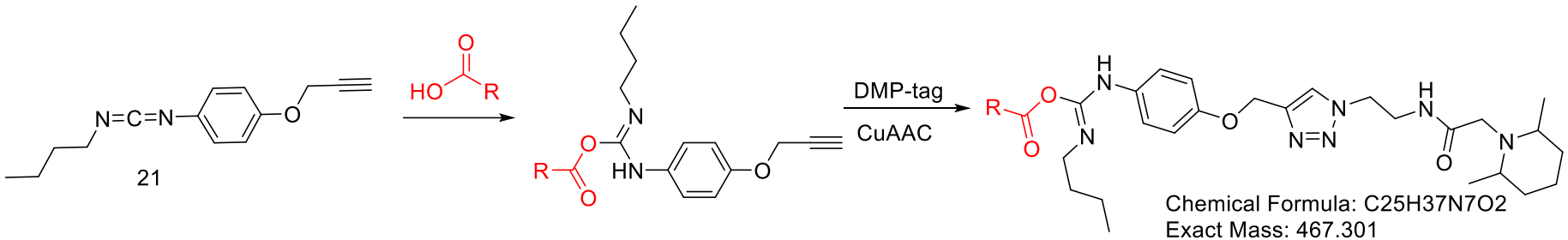
DMP-tag
CuAAC
Chemical Formula: C27H32ClN7O2
Exact Mass: 521.231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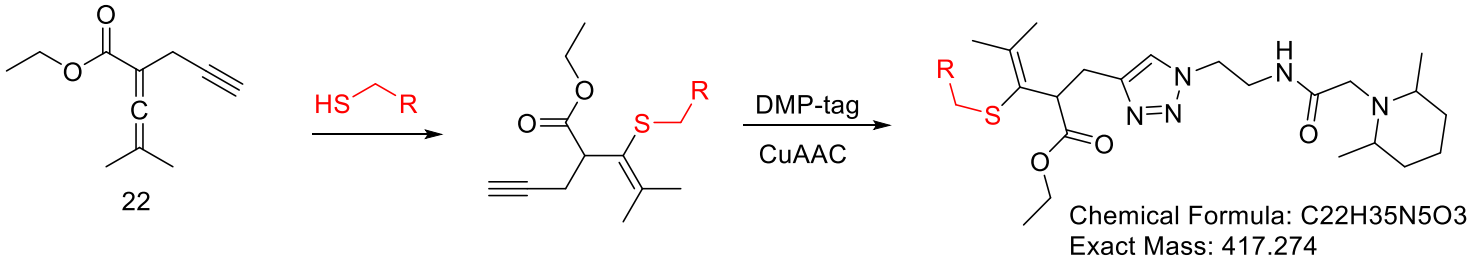
DMP-tag
CuAAC
Chemical Formula: C25H37N7O2
Exact Mass: 467.301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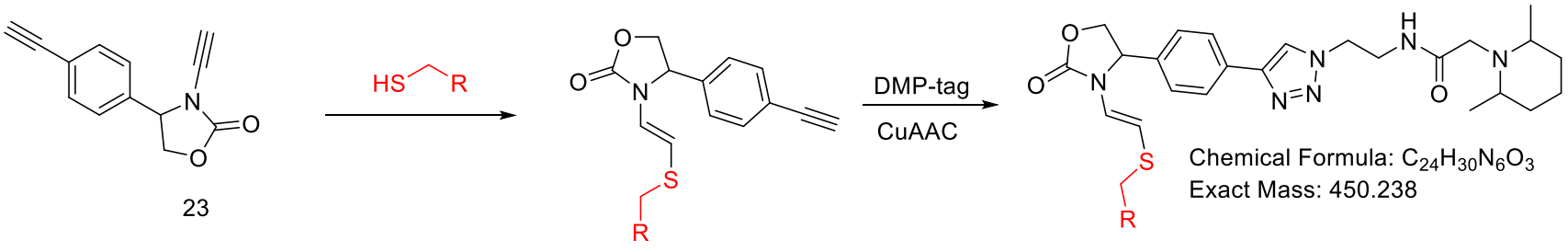
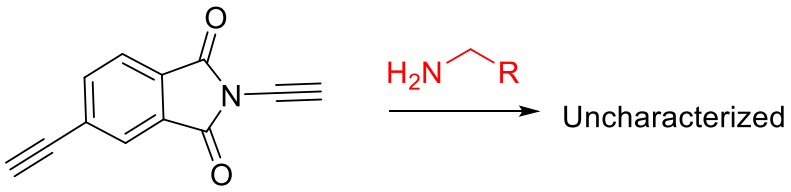
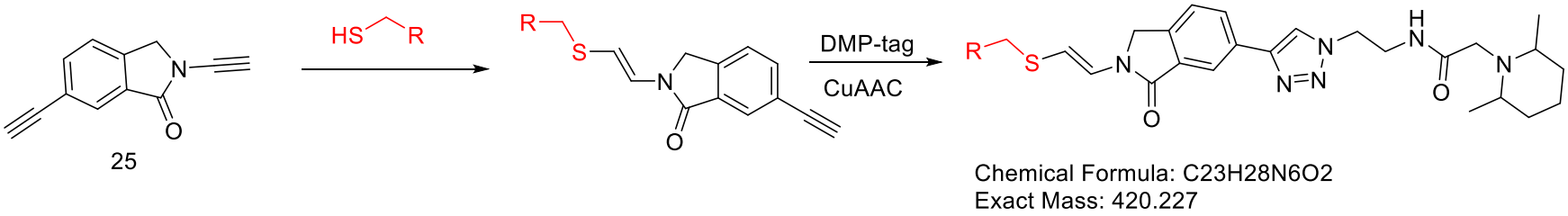
DMP-tag
CuAAC
Chemical Formula: C22H35N5O3
Exact Mass: 417.274


HS
R
DMP-tag
CuAAC
Chemical Formula: $C_{24}H_{30}N_6O_3$
Exact Mass: 450.238

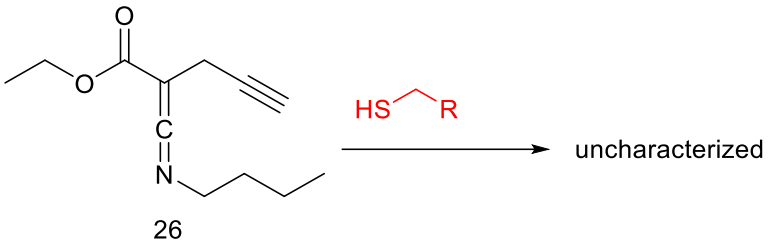
23


H_2N
R
Uncharacterized

24


HS
R
DMP-tag
CuAAC
Chemical Formula: C23H28N6O2
Exact Mass: 420.227

25



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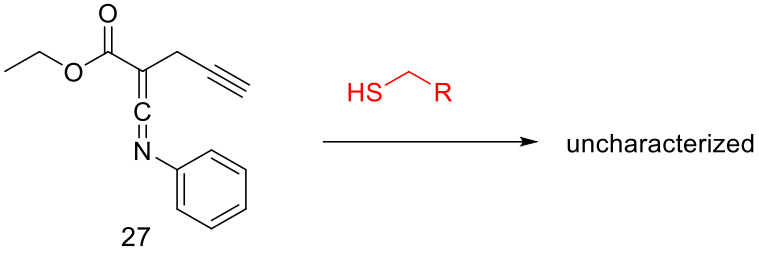

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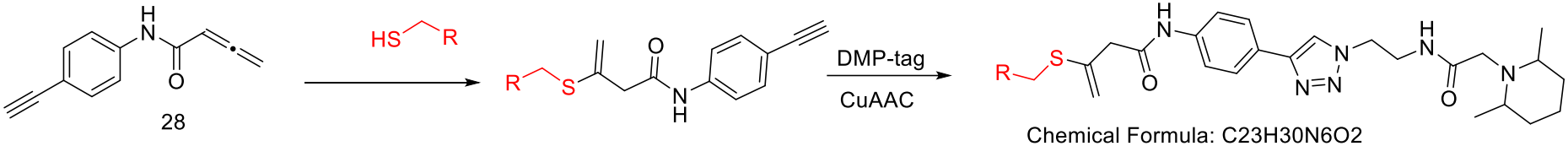

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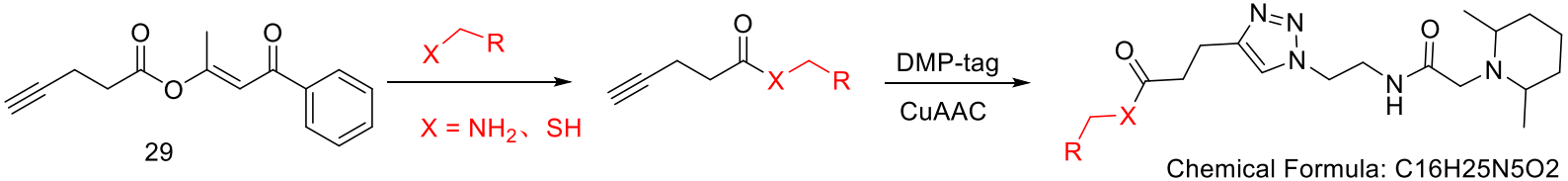
Chemical Formula: C23H30N6O2
Exact Mass: 422.243



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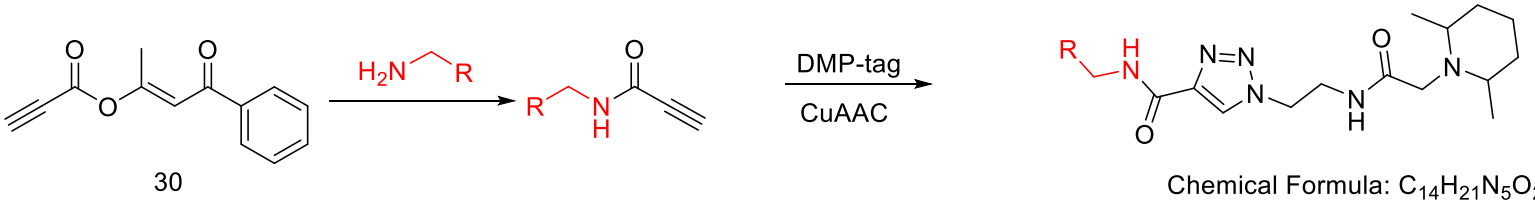
Chemical Formula: C16H25N5O2
Exact Mass: 319.201



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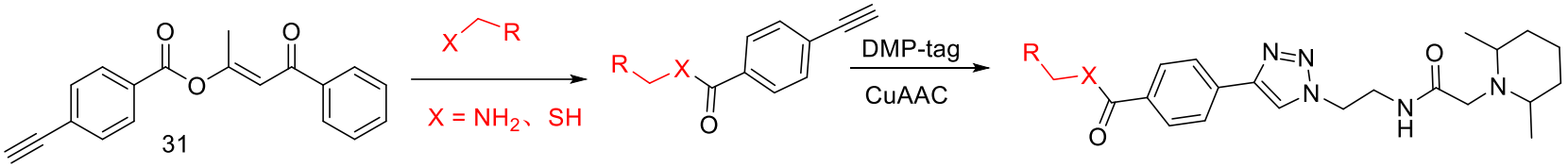
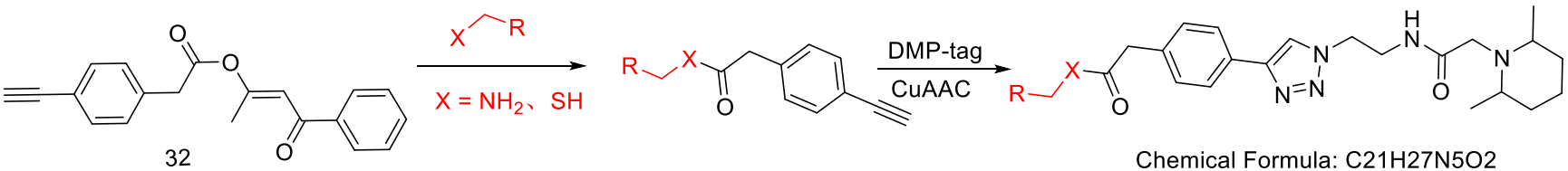
Chemical Formula: $C_{14}H_{21}N_5O_2$
Exact Mass: 291.170



31

Chemical Formula: $C_{20}H_{25}N_5O_2$
Exact Mass: 367.201



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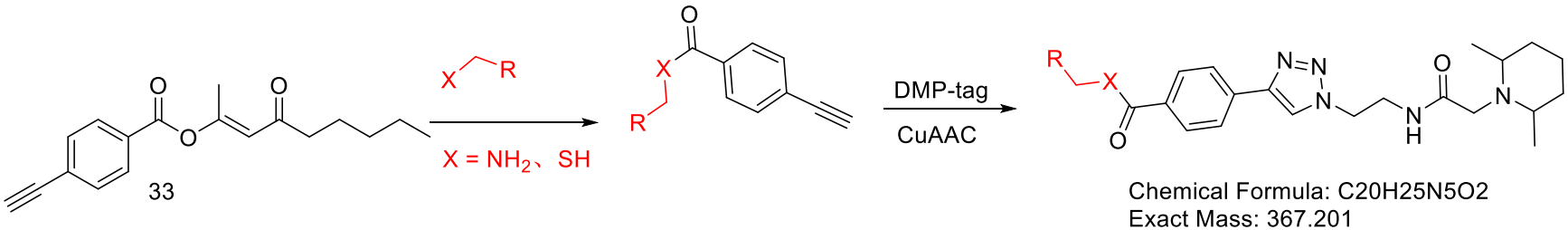
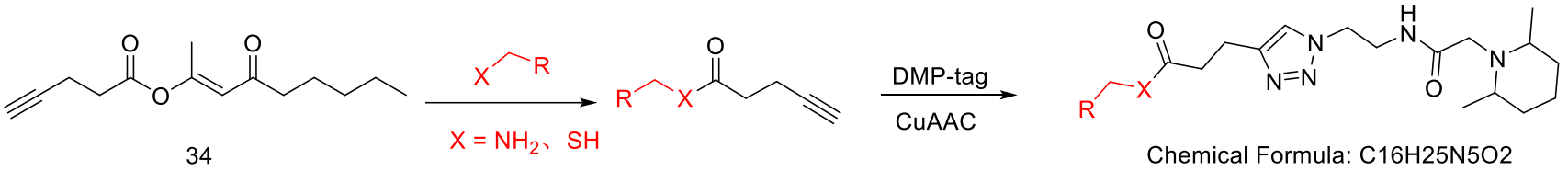
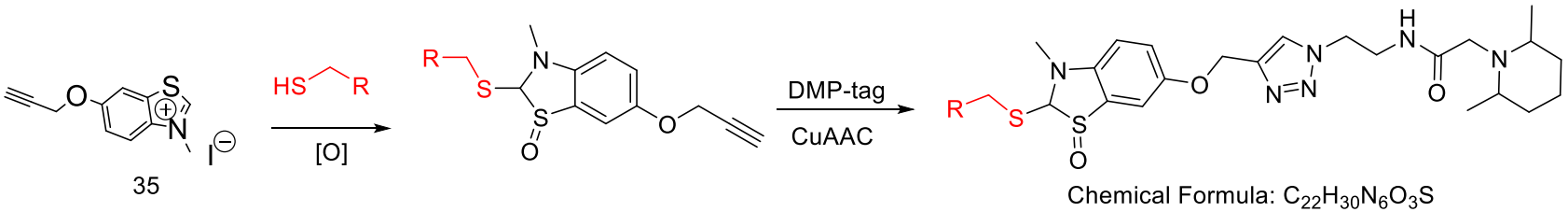
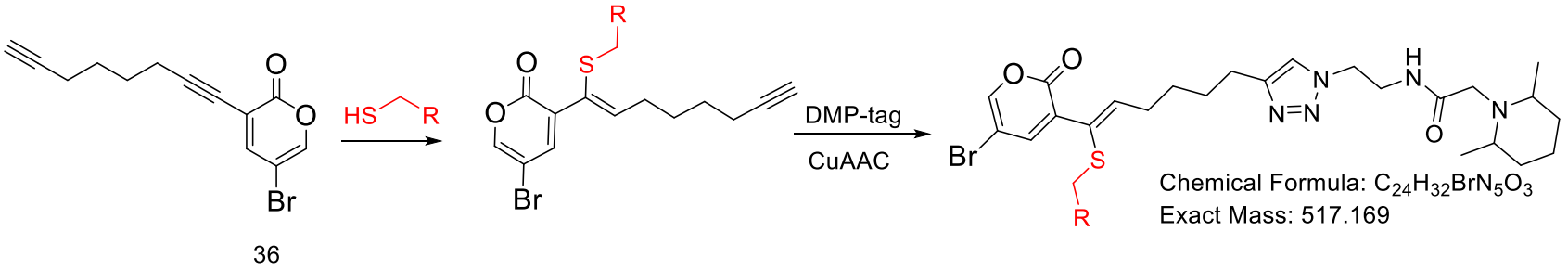


33

Chemical Formula: C20H25N5O2
Exact Mass: 367.201


34
X = NH2、SH
DMP-tag
CuAAC
Chemical Formula: C16H25N5O2
Exact Mass: 319.201


35
[O]
DMP-tag
CuAAC
Chemical Formula: C22H30N6O3S
Exact Mass: 458.210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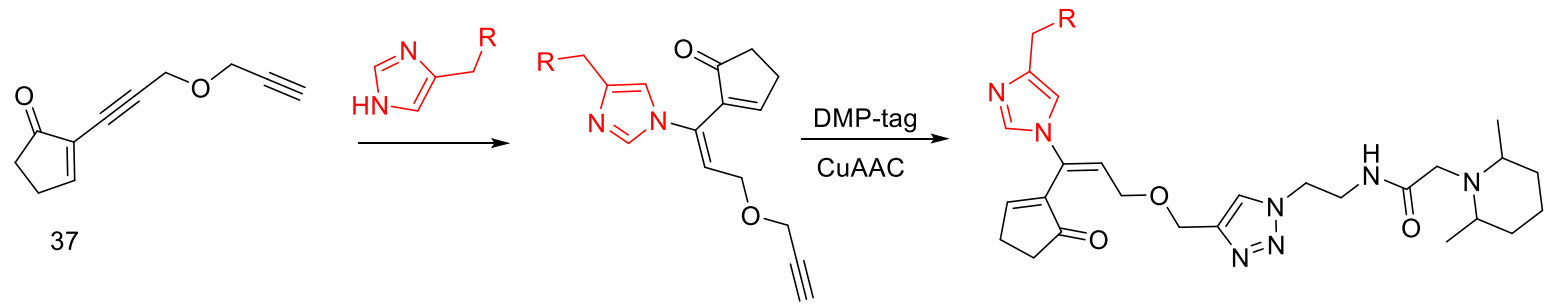
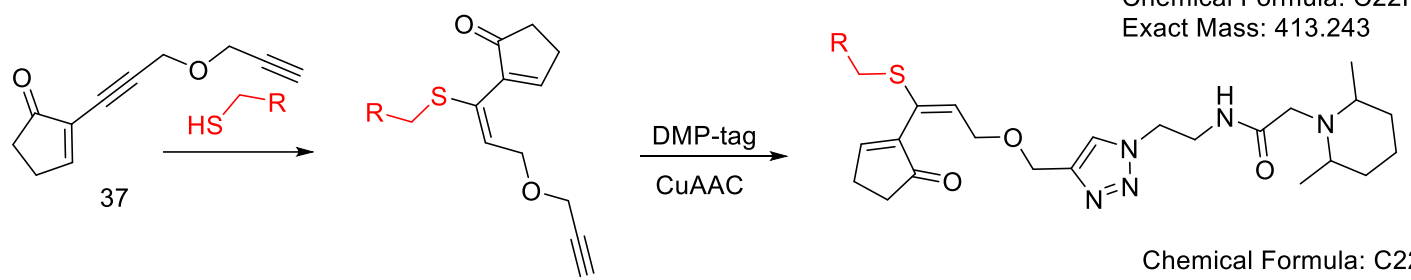
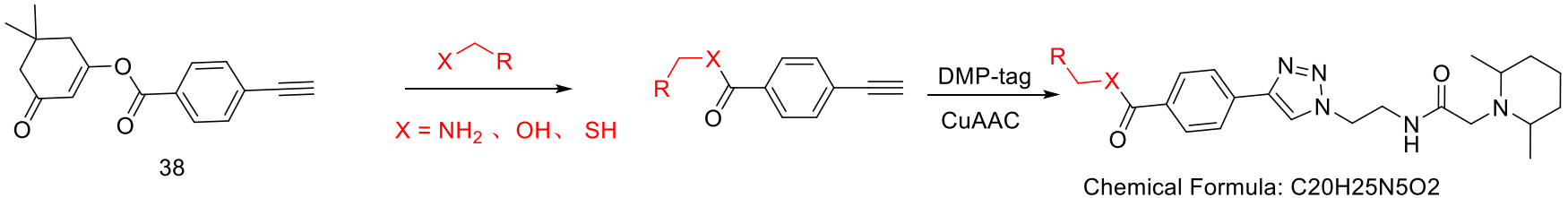
DMP-tag
CuAAC
Chemical Formula: C24H32BrN5O3
Exact Mass: 517.169



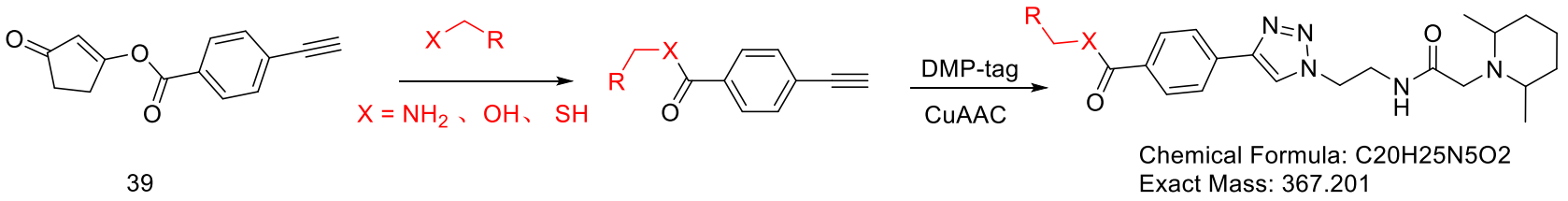
Chemical Formula: C22H31N5O3
Exact Mass: 413.243



Chemical Formula: C22H31N5O3
Exact Mass: 413.243



Chemical Formula: C20H25N5O2
Exact Mass: 367.201



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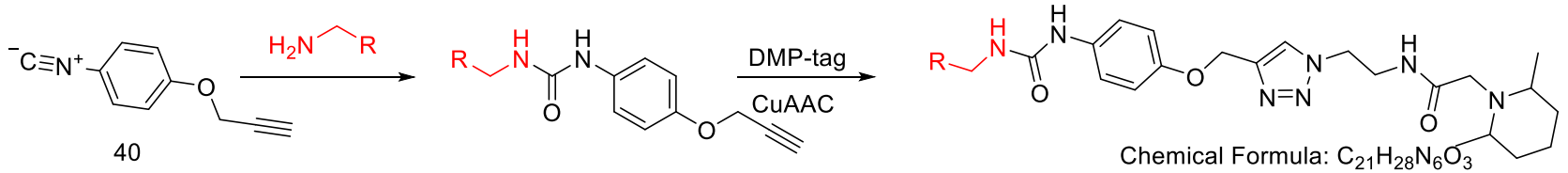
Chemical Formula: C20H25N5O2
Exact Mass: 367.201



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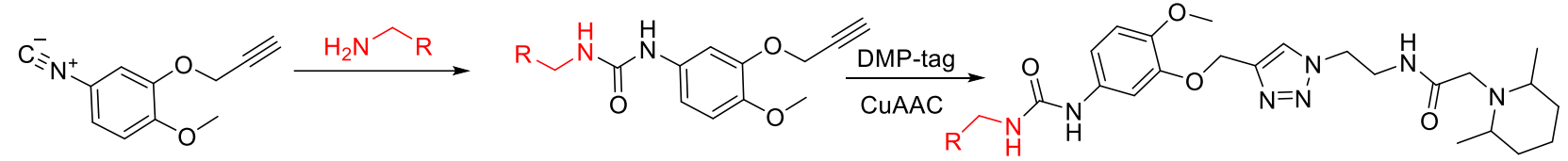
Chemical Formula: $C_{21}H_{28}N_6O_3$
Exact Mass: 412.222



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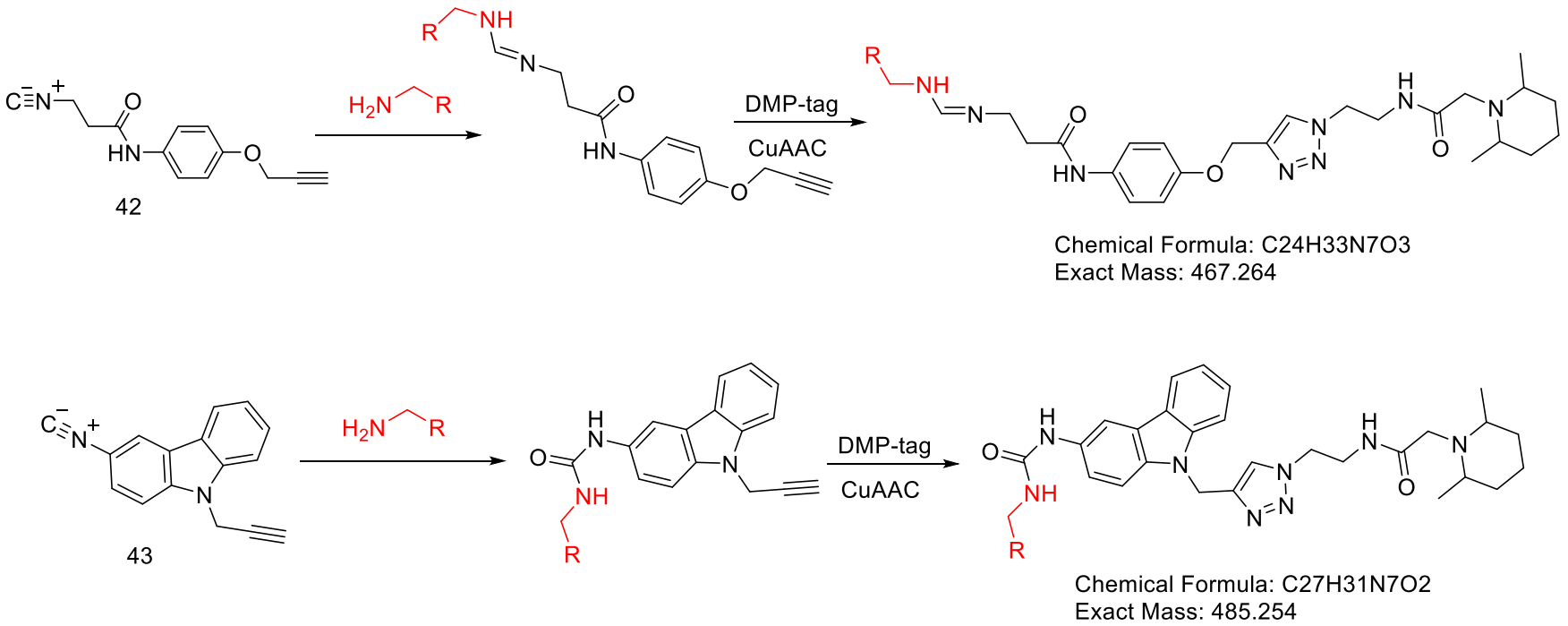
Chemical Formula: C22H30N6O4
Exact Mass: 442.233



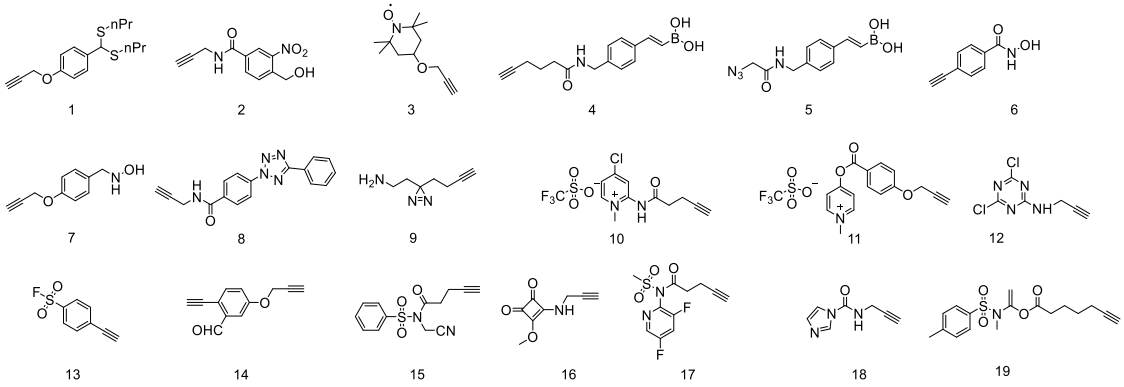
Supplementary Fig. 9. Bioconjugation reactions and resulting probe-derived modifications (PDMs). Schematic representation of the bioconjugation reactions between the indicated probes and their target amino acid residues revealed by plon search. The corresponding DMP-tagged end products (PDMs) are shown alongside their theoretical masses. It is important to note that these proposed chemical structures are presented as plausible hypotheses, where further mechanistic work is needed.

Chemical synthesis

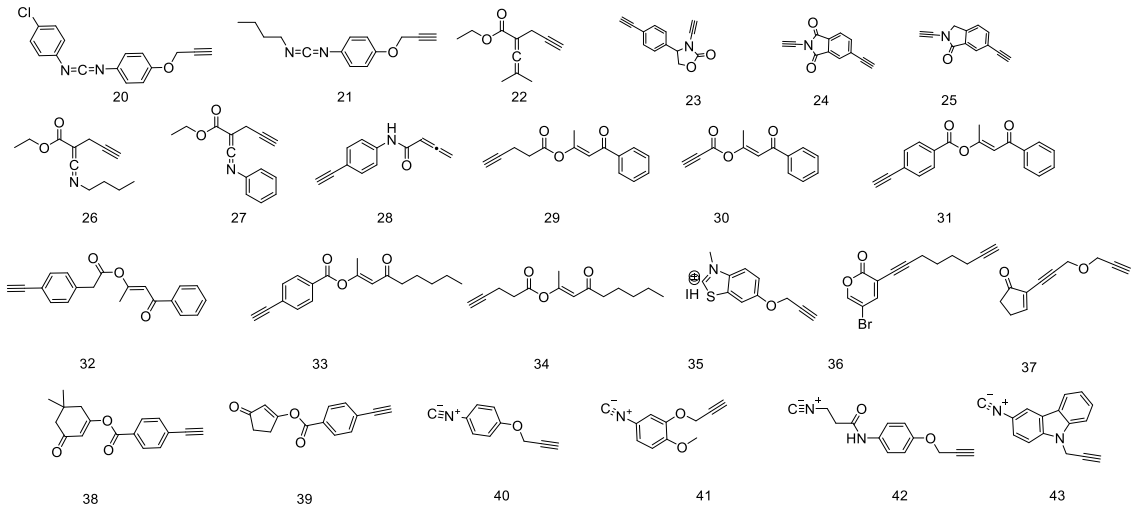
1. General materials

All reactions were carried out under an atmosphere of argon in oven-dried glassware with a magnetic stirring bar**,** all chemicals were purchased from commercial vendors and used without further purification, unless indicated otherwise. All reactions requiring anhydrous conditions were carried out under argon or nitrogen atmosphere using oven-dried glassware. AR-grade solvents were used for all reactions. Reaction progress was monitored by TLC on pre-coated silica plates (HSG F254 nm, 0.25 μm) and spots were visualized by UV, iodine or other suitable stains. Flash column chromatography was carried out using silica gel (HSG F254 nm, 0.040-0.063 μm). All NMR spectra (^{1}H-NMR, ^{13}C-NMR) were recorded on Bruker Vance ARX- 400. Chemical shifts were reported in parts per million (ppm) referenced with respect to appropriate internal standards or residual solvent peaks ($CDCl_3$ = 7.26 ppm, DMSO-*d6* = 2.50 ppm). The following abbreviations were used in reporting spectra, br s (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). Mass spectra were obtained on Agilent LC-ESI-MS system. All analytical HPLC were carried out on Agilent system.

2. Structure of reported diverse chemical probes

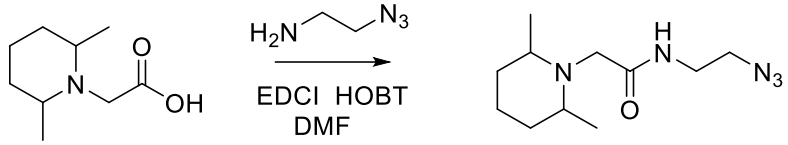


3. Structure of newly synthesized chemical probes

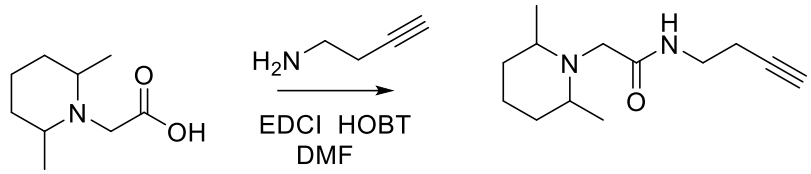


4. Synthetic procedures of DMP tag

DMP-tag1



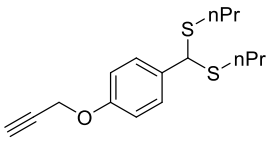
N-(2-azidoethyl)-2-(2,6-dimethylpiperidin-1-yl)acetamide(DMP-tag1) DMP-tag1 was reported and synthesis of **DMP-tag1** was carried out similar to literature procedure[18] 2-(2,6-dimethylpiperidin-1-yl)acetic acid (171 mg, 1 mmol), HOBT (148 mg, 1.1 mmol), EDC.HCl (210 mg, 1.1 mmol) and DIPEA (387 mg, 3 mmol) were dissolved in anhydrous DMF (15 mL). The mixture was stirred for 3 h at r.t. The mixture of azide 11 (75.9 mg, 1.1mmol) with DIPEA (129 mg, 1 mmol) in anhydrous DMF (5 mL) was added to the mixture. The mixture was stirred under rt for 72 h at r.t. The mixture was washed with H_2O (15 mL) and brine (1 × 15 mL). The organic phase was dried over anhydrous Mg_2SO_4 and the solvent was removed in vacuo. The product was purified by column chromatography using 5% MeOH in DCM as eluent. Product was obtained as a yellow liquid (0.16 g, 70%),purified 97.8% .^{1}H NMR (400 MHz, DMSO) δ 7.94 (s, 1H), 3.38 (t, *J* = 5.7 Hz, 2H), 3.31 (dd, *J* = 10.5, 4.5 Hz, 3H), 2.98 (s, 2H), 2.48 – 2.39 (m, 2H), 1.64 – 1.57 (m, 1H), 1.57 – 1.45 (m, 2H), 1.26 (dt, *J* = 21.3, 12.0 Hz, 3H), 0.98 (d, *J* = 6.3 Hz, 7H). HRMS (ESI) calcd.for $C_{13}H_{22}N_2O$ 239.1750 $[M + H]^+$, found 240,1720



N-(but-3-yn-1-yl)-2-(2,6-dimethylpiperidin-1-yl)acetamide(DMP-tag2) DMP-tag2 was reported and synthesis of DMP-tag2 was carried out similar to literature procedure[18]. 2-(2,6-dimethylpiperidin-1-yl)acetic acid (171 mg, 1 mmol), HOBT (148 mg, 1.1 mmol), EDC.HCl (210 mg, 1.1 mmol) and DIPEA (387 mg, 3 mmol) were dissolved in anhydrous DMF (15 mL). The mixture was stirred for 3 h at r.t.. The mixture of azide 11 (94.6 g, 1.1mmol) with DIPEA (129 mg, 1 mmol) in anhydrous DMF (5 mL) was added to the mixture. The mixture was stirred under rt for 72 h at r.t. The mixture was washed with H_2O (15 mL) and brine (1 × 15 mL). The organic phase was dried over anhydrous Mg_2SO_4 and the solvent was removed in vacuo. The product was purified by column chromatography using 5% MeOH in DCM as eluent. Product was obtained as a yellow liquid (0.13 g, 60%) purified 97.8% .^{1}H NMR (400 MHz, DMSO).δ 7.91 (s, 1H), 3.23 (q, J = 6.7 Hz, 2H), 2.96 (s, 2H), 2.84 (t, J = 2.6 Hz, 1H), 2.47 – 2.40 (m, 2H), 2.30 (td, J = 6.8, 2.6 Hz, 2H), 1.66 – 1.44 (m, 3H), 1.27 (dddd, J = 23.8, 14.6, 9.7, 3.3 Hz, 3H), 0.96 (t, J = 9.2 Hz, 6H) HRMS (ESI) calcd.for $C_{13}H_{22}N_2O$ 222.1730 $[M + H]^+$, found 223,1700

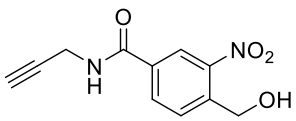
5. These compounds were synthesized and applied in DMP-tag-based pipeline

1



((4-(prop-2-yn-1-yloxy)phenyl)methylene)bis(propylsulfane)(1) The compound was aquired from Li group. Related compond structure and experiments were carried out according the reference[19], light yellow oil, yield 91%.

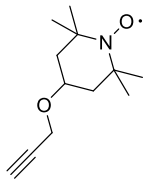
2



4-(hydroxymethyl)-3-nitro-N-(prop-2-yn-1-yl)benzamide(2) The compound C4 was synthesized according to published literature[20] ^{1}H NMR (400 MHz, DMSO) δ 9.33 – 9.20 (m, 1H), 8.53 (d, J = 2.0

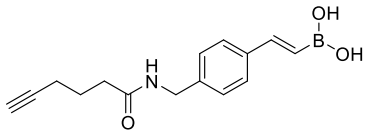
Hz, 1H), 8.24 (dd, J = 8.5, 1.8 Hz, 1H), 7.95 (d, J = 8.7 Hz, 1H), 5.68 (t, J = 5.9 Hz, 1H), 4.88 (d, J = 5.9 Hz, 2H), 4.10 (dd, J = 5.9, 2.7 Hz, 2H), 3.20 – 3.01 (m, 2H). 13C NMR (151 MHz, DMSO) δ 164.29 (s), 147.03 (s), 142.15 (s), 133.62 (s), 132.64 (s), 129.03 (s), 123.69 (s), 81.34 (s), 73.67 (s), 60.37 (s), 29.16 (s).

3



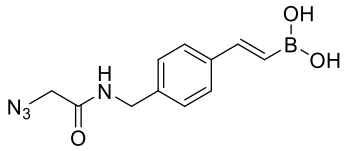
(3) The compound was purchased from Bide Pharmaceuticals purify 95% (CAS: 147045-24-7, Cat. No BD01096089). Related compond structure and experiments were carried out according the reference[21].

4



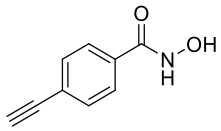
(E)-(4-(hex-5-ynamidomethyl)styryl)boronic acid (4) The compound was aquired and synthesised from Dang group, and related biological experiments were carried out according the reference[14].

5



(E)-(4-((2-azidoacetamido)methyl)styryl)boronic acid(5) The compound was aquired and synthesised from Dang group and related biological experiments were carried out according the reference[14].

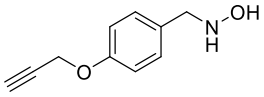
6



4-ethynyl-N-hydroxybenzamide (6) The compound was synthsized according to the literature reported [22] ^{1}H NMR (400 MHz, DMSO) δ 11.31 (s, 1H), 9.12 (s, 1H), 7.75 (d, J = 7.8 Hz, 2H), 7.55 (d,

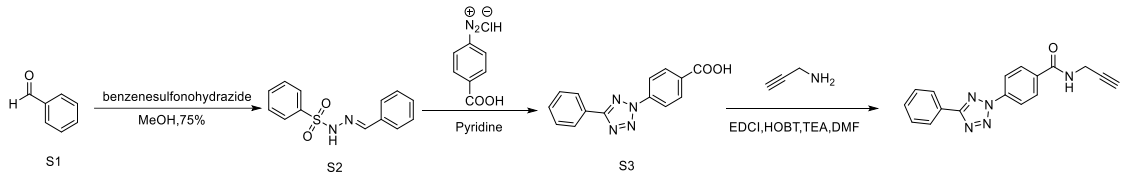
J = 7.8 Hz, 2H), 4.35 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 163.86, 133.33, 132.20, 127.63, 124.85, 83.30, 83.23. ESI-MS calcd for $C_9H_7NO_2$ [M+H]$^+$ 162.0550; Found 162.0551.

7



N-(4-(prop-2-yn-1-yloxy)benzyl)hydroxylamine (7) The compound was obtained and synthesised from Li group , and related biological experiments were carried out according the reference[23] .

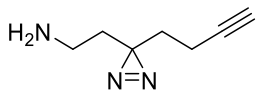
8



4-(5-phenyl-2H-tetrazol-2-yl)-N-(prop-2-yn-1-yl)benzamide (8) .Synthesis of the 26 was based on previously published procedures[24]. To a suspension of 4-aminobenzoic acid (121 mg, 1 mmol) in 5 mL H_2O and 5 mL EtOH was added 0.5 mL concentrated HCl, followed by addition of a solution of $NaNO_2$ (69 mg, 1 mmol) in 1 mL H_2O at 0 °C. After stirring for 10 min, the solution was added dropwise to the solution of **S2** (1 mmol) in 5 mL pyridine over 50 min at -10 °C. The mixture was warmed up to rt. prior to addition of 30 mL H_2O. The solution was extracted with 2 × 10 mL of ethyl acetate, the combined organic layers were washed successively with 3×100 mL 2 N HCl and 70 mL brine. Upon solvent evaporation *in vacuo*, the residue was purified by flash column (PE: EA = 1:1, R_f = 0.2) to give **S3** as a brown solid (200 mg ,60%). To a stirred solution of S3 (40 mg, 0.15 mmol) in 5 mL of DMF was added HOBT (41 mg, 0.3 mmol), EDCI (58 mg, 0.3 mmol) and TEA (30 mg, 0.3 mmol). The mixture was stirred for 30 min at r.t followed by addition of propargylamine (9 mg, 0.3 mmol). The reaction was then stirred at room temperature overnight in the dark. Subsequently, it was quenched by addition of 5 mL water and extracted with 2 × 10 mL of ethyl acetate. The organic layers were washed with 2×10 mL brine and dried over anhydrous Na_2SO_4 Upon solvent evaporation in vacuo, the residue was purified by flash column (PE : EA = 5:1) to give Tz1 as a white solid **8**(38 mg, 83%), R_f = 0.6 (PE:EA = 1:1)^{1}H NMR (400 MHz, DMSO-d_6) δ 9.24 (d, J = 5.5 Hz, 1H), 8.32 – 8.24 (m, 3H), 8.22 – 8.13 (m, 5H), 7.66 – 7.55 (m, 4H), 4.11 (dd, J = 5.5, 2.5 Hz, 2H), 3.20 (t, J = 2.5 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.12 (d, J = 2.7 Hz), 138.35, 135.36, 131.58, 129.81 (d,

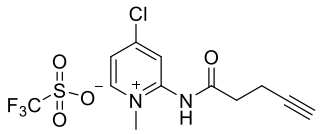
J = 10.8 Hz), 127.17, 126.70, 120.17, 81.53, 73.62, 29.12. HR-MS (ESI) Calcd $C_{17}H_{13}N_5O$ for $[M+H]^+$ 304.1198; found 304.1193.

9



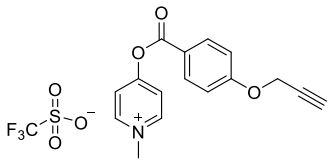
2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine(9) The compond was purchased from Bide Pharmaceuticals purify 95% (CAS: 1450752-97-2, Cat.No BD627887) and related biological experiments were carried out according the reference[25].

10



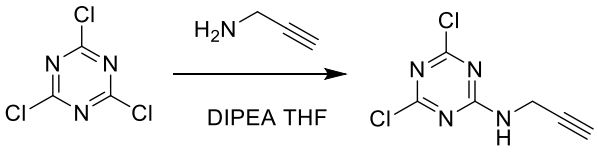
4-chloro-1-methyl-2-(pent-4-ynamido)pyridin-1-ium-trifluoromethanesulfonate salt（10） The compound was borrowed and synthesised from Li group , and related biological experiments were carried out according the reference [26].

11



1-methyl-4-((4-(prop-2-yn-1-yloxy)benzoyl)oxy)pyridin-1-ium-trifluoromethane -sulfonate salt（11）The compound was borrowed and synthesised from Li group , and related biological experiments were carried out according the reference [27].

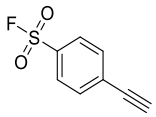
12



4,6-dichloro-N-(prop-2-yn-1-yl)-1,3,5-triazin-2-amine(12) Propargylamine (0.59 g, 0.69 mL, 10.76 mmol) was added to a stirred solution of cyanuric chloride (2.00 g, 10.85 mmol) in dry THF (25 mL) cooled to -20 °C. DIPEA (1.54 g, 2.08 mL, 11.94 mmol) in dry THF (5 mL) was then added dropwise over a period of 2 h with the help of a syringe pump. Thereafter, reaction mixture was stirred for another

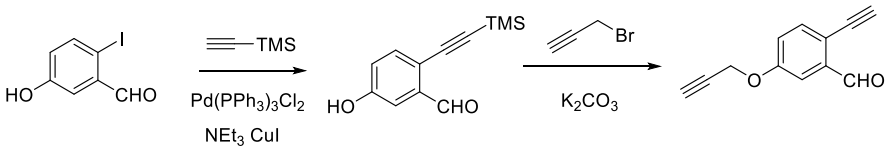
3 h maintaining the reaction temperature between -20 and 0 °C. After this time, THF was removed under vacuum to yield a residue that was dissolved in EtOAc (50 mL) in a separating funnel and washed with water (3 × 15 mL), followed by brine solution (30 mL) and finally dried over solid anhydrous Na_2SO_4 to yield a pure product (white precipitate) in quantitative yield (2.18 g, yield 99%) under reduced pressure ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.74 (s, 1H), 4.30 (dd, *J* = 5.8, 2.5 Hz, 2H), 2.31 (t, *J* = 2.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.73 (s), 169.65 (s), 165.00 (s), 77.18 (s), 72.31 (s), 30.76 (s). HRMS (ESI) calcd.for $C_6H_4Cl_2N_4$ 202.9875 $[M + H]^+$, found 202.9885.

13



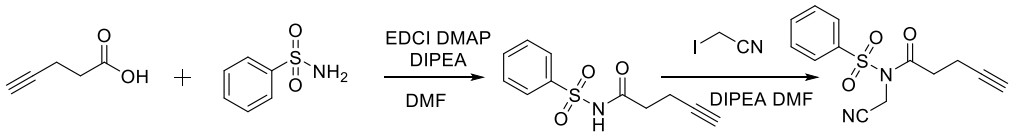
4-ethynylbenzenesulfonyl fluoride(13) The compound was purchased from Bide Pharmaceuticals （NO.BD01353772）, purity 95% ^{1}H NMR (400 MHz, DMSO) δ 8.13 (d, *J* = 2.0 Hz, 2H), 7.86 (d, *J* = 10.3 Hz, 2H), 4.75 (d, *J* = 2.9 Hz, 1H).^{13}C NMR (101 MHz, $CDCl_3$) δ 133.12 (s), 132.81 (s), 132.55 (s), 129.98 (s), 128.41 (s), 82.75 (s), 81.27 (s).

14



2-ethynyl-5-(prop-2-yn-1-yloxy)benzaldehyde(14) Related compond were synthsised according the reference reported[28] ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.51 (s, 1H), 6.81 (d, J = 12.3 Hz, 1H), 6.56 (d, J = 3.1 Hz, 1H), 6.48 (dd, J = 9.1, 3.0 Hz, 1H), 4.10 (s, 2H), 2.80 (s, 1H), 1.67 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 191.16 (s), 158.08 (s), 137.87 (s), 135.83 (s), 122.30 (s), 118.25 (s), 112.39 (s), 86.78 (s), 79.46 (d, J = 2.2 Hz), 78.94 (s), 56.37 (s). HRMS (ESI) calcd. for $C_{12}H_8O_2$ 185.0597 $[M+H]^+$, found 185.0587.

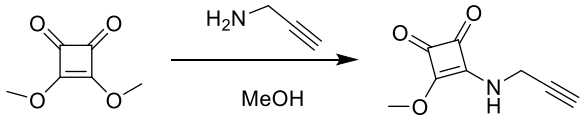
15



N-(cyanomethyl)-N-(phenylsulfonyl)pent-4-ynamide(15) Related compond were synthsised according the reference reported[13] ^{1}H NMR (400 MHz, DMSO) δ 8.05 (d, J = 10.0 Hz, 1H), 7.86 – 7.79

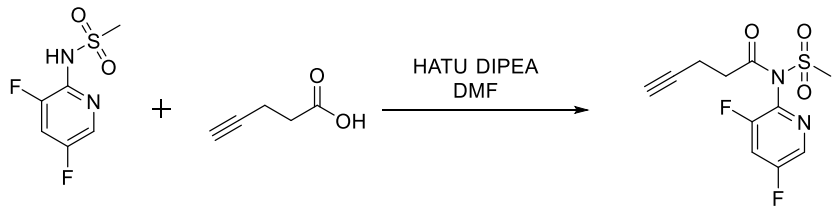
(m, 1H), 7.72 (d, J = 9.4 Hz, 1H), 4.96 (s, 2H), 2.93 (d, J = 8.5 Hz, 1H), 2.76 (s, 1H), 2.42 – 2.28 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 170.91 (s), 138.20 (s), 135.38 (s), 130.27 (s), 128.19 (s), 116.67 (s), 83.27 (s), 72.13 (s), 35.10 (s), 34.31 (s), 13.70 (s). HRMS (ESI) calcd. for $C_{13}H_{12}N_2O_3S$ 299.0460 $[M+Na]^+$, found 299.0444.

16



3-methoxy-4-(prop-2-yn-1-ylamino)cyclobut-3-ene-1,2-dione(16) To a stirred solution of 3,4-dimethoxycyclobut-3-ene-1,2-dione (300 mg, 2.1 mmol) was added in the in MeOH(5.0 mL), followed by the Propargylamine(104 mg, 1.9 mmol) were added. The mixture was stirred at room temperature at 2 h, The product precipitates directly in methanol, filtrated from MeOH to gain a white solid (303 mg, yelid 75%) ^{1}H NMR (400 MHz, DMSO) δ 9.06 (s, 1H), 4.29 (s, 3H), 4.07 – 3.81 (m, 1H), 3.35 (d, J = 20.7 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 189.62 (s), 183.35 (s), 178.52 (s), 172.72 (s), 80.46 (s), 76.02 (s), 60.79 (s), 33.57 (s). HRMS (ESI) calcd. for $C_8H_7NO_3$ 166.0498 $[M+H]^+$, found 166.0489.

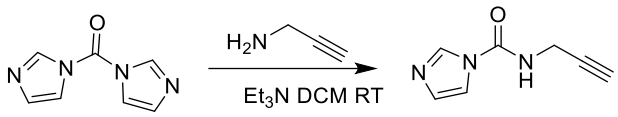
17



N-(3,5-difluoropyridin-2-yl)-N-(methylsulfonyl)pent-4-ynamide(17) To a stirred solution of N-(3,5-difluoropyridin-2-yl)methanesulfonamide (50 mg, 0.24 mmol) was added in the in DMF(5.0 mL),then HATU(91 mg, 0.24 mmol) and DIPEA(38.7 mg, 0.3 mmol) were added into the mixture. After 5min, followed by the Propargylamine(19.6 mg, 0.2 mmol) were added. The mixture was stirred at room temperature overnight, followed by addition of water (10.0 mL). The mixture was extracted with ethyl acetate, and the combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by flash chromatography to gain a white solid (31.8 mg, yelid 45%). ^{1}H NMR (400 MHz, DMSO) δ 8.05 (d, J = 10.0 Hz, 1H), 7.81 (d, J = 8.8 Hz, 1H), 7.71 (t, J = 9.1 Hz, 1H), 4.96 (s, 2H), 3.03 – 2.85 (m, 1H), 2.76 (s, 1H), 2.44 – 2.25 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 173.30 (s), 137.78 (s), 131.44 (s), 114.38 (s), 114.25 (s), 114.09 (s), 83.92 (s),

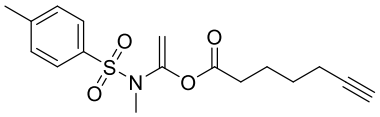
71.82 (s), 43.01 (s), 33.35 (s), 14.28 (s). HRMS (ESI) calcd. for $C_{11}H_{10}F_2N_2O_3S$ 311.0272 $[M+Na]^+$, found 311.0251.

18



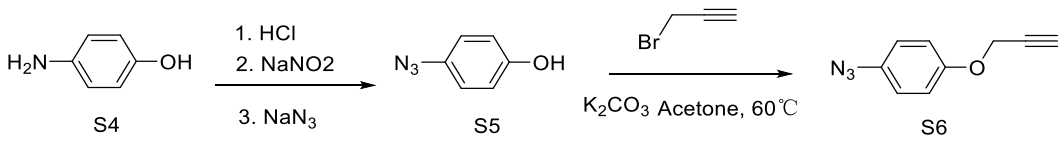
N-(prop-2-yn-1-yl)-1H-imidazole-1-carboxamide(18) CDI (1.7 g, 11 mmol, 1.10 eq) and propargyl amine (0.55 g, 10 mmol, 1.0 eq) were dissolved in DMF (5 mL) and acetonitrile (6 mL). The solution was stirred at room temperature for 2 h give a white solid (1.59 g, yield 71%) ^{1}H NMR (600 MHz, DMSO) δ 9.02 (s, 1H), 8.26 (s, 1H), 7.68 (s, 1H), 7.04 (s, 1H), 4.08 (s, 2H), 3.25 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 148.80 (s), 135.92 (s), 129.88 (s), 116.70 (s), 78.54 (s), 72.47 (s), 30.57 (s). HRMS (ESI) calcd $C_7H_7N_3O$ 150.0661 for $[M+H]^+$ found 150.0654.

19



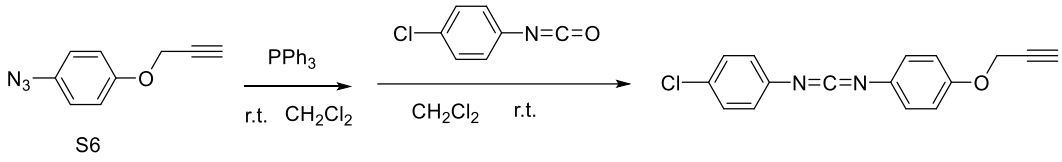
1-((N,4-dimethylphenyl)sulfonamido)vinyl hept-6-ynoate(19) Related compound was synthesized according the reference reported [11] and gained from the LZQ groups ^{1}H NMR (400 MHz, CDCl3) δ 7.78 – 7.65 (m, 2H), 7.33 (d, J = 8.1 Hz, 2H), 4.81 (d, J = 2.6 Hz, 1H), 4.62 (d, J = 2.6 Hz, 1H), 3.01 (s, 3H), 2.44 (s, 3H), 2.40 – 2.29 (m, 2H), 2.20 (m, 2H), 1.96 (t, J = 2.6 Hz, 1H), 1.78 – 1.63 (m, 2H), 1.63 – 1.47 (m, 2H). 13C NMR (151 MHz, CDCl3) δ 170.8, 147.0, 144.1, 133.8, 130.0, 129.6, 129.6, 129.5, 128.0, 128.0, 128.0, 127.3, 100.4, 83.8, 68.8, 37.3, 33.4, 27.6, 23.5, 21.6, 18.1. ESI-MS calcd for C17H21NO4S [M+Na]+ m/z = 358.1084; Found 358.1074.

20



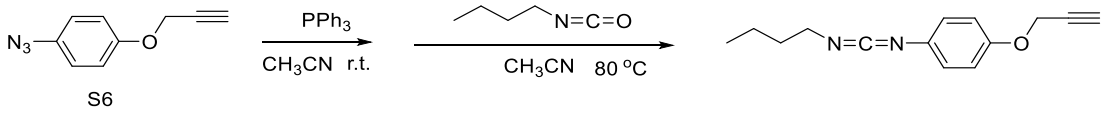
1-azido-4-(prop-2-yn-1-yloxy)benzene (S6) To a mixture of 4-aminophenol **S4** (0.01mol) and 15% HCl (10mL), $NaNO_2$ (0.012mol) in H_2O (30mL) was added drop-wise at 0°C. After the completion of addition, the reaction mixture was stirred at this temperature for 30min. A solution of sodium azide (0.02mol in 10mL H_2O) was added dropwise to the reaction mixture at 0°C. After addition the reaction

mixture was maintained at 0°C for 4h. The product was extracted by using ethyl acetate followed by washing with water up to neutral pH. Organic layer was dried with anhydrous sodium sulfate, filtered and concentrated under vacuum to afford product S**5**, which was used as the starting material in the next step without further purification and characterization. To a solution of crude azide **S5** in dry acetone (30 mL) was added the propargyl bromide (11 mmol) and K_2CO_3 (40 mmol) at r.t., then the mixture was stirred at 60 °C for 12 h, when the reaction was complete, the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc/petroleum ether, 1:10) to give product **S6,** the synthesized compound **S6** using the next step for starting material.



***N*-(4-chlorophenyl)-*N*-(4-(prop-2-yn-1-yloxy)phenyl)methanediimine(20):**To a solution of azide **S6** (2 mmol) in CH_2Cl_2 (10 mL) was added PPh_3 (2 mmol), the mixture was stirred at room temperature for 1 h (TLC monitoring). Then *p*-chlorophenyl isocyanate (2 mmol) was added at rt., and the reaction mixture was stirred at room temperature for 6 h. when the reaction was complete, the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc/petroleum ether = 1:10) to give product **20**. yellow solid (0.475g, 84% yield), mp 155-157°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 7.28-7.26 (m, 2H), 7.13-7.08 (m, 4H), 6.95-6.93 (m, 2H), 4.68 (d, *J* = 2.4 Hz, 2H), 2.53 (t, *J* = 2.4 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 155.5, 137.5, 135.3, 131.1, 130.8, 129.5, 125.3, 125.2, 115.9, 78.2, 75.8, 56.1. HRMS (ESI) calcd.for $C_{14}H_{16}N_2O$ 283.0614 [M + H]$^+$, found. 283.0617.

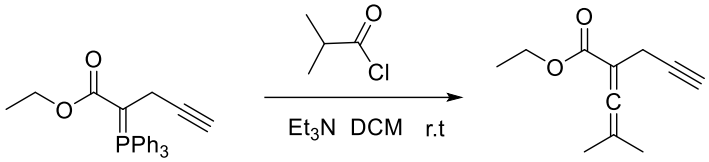
21



***N*-butyl-*N*-(4-(prop-2-yn-1-yloxy)phenyl)methanediimine (21):** To a solution of azide **S6** (2 mmol) in CH_3CN (10 mL) was added PPh_3 (2 mmol), the mixture was stirred at room temperature for 1 h (TLC monitoring). Then *n*-butyl isocyanate (2 mmol) was added at r.t, and the reaction mixture was

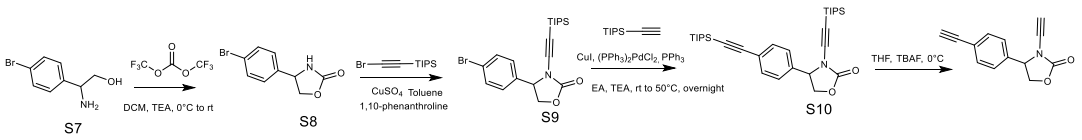
stirred at 80 °C for 8 h. when the reaction was complete, the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc/petroleum ether, 1:10) to give product **21**. yellow oil (0.365g, 80% yield); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 7.04-7.01 (m, 2H), 6.92-6.89 (m, 2H), 4.66 (d, *J* = 2.4 Hz, 2H), 3.39 (t, *J* = 6.8 Hz, 1H), 3.39 (t, *J* = 6.8 Hz, 1H), 2.52 (t, *J* = 6.8 Hz, 1H), 1.69-1.62 (m, 2H), 1.47-1.42 (m, 2H), 0.95 (t, *J* = 7.6 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 154.6, 136.7, 134.2, 124.3, 115.8, 78.5, 75.5, 56.1, 46.6, 33.4, 19.9, 13.5. HRMS (ESI) calcd.for $C_{14}H_{16}N_2O$ 229.1335 [M + H] $^{+}$, found. 229.1331.

22



Ethyl 4-methyl-2-(prop-2-yn-1-yl)penta-2,3-dienoate (22): To a solution of phosphorane (2 mmol) in dry CH_2Cl_2 (10 mL) was added Et_3N (3 mmol), then isobutyryl chloride (2 mmol) was added at 0 ℃. After the addition, the mixture was stirred for 24 h at room temperature. when the reaction was complete, the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc/petroleum ether, 1:10) to give product **22**. yellow oil (0.278g, 78% yield); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 4.22-4.16 (m, 4H), 3.14 (d, *J* = 2.4 Hz, 2H), 2.04 (t, *J* = 2.4 Hz, 1H), 1.82 (s, 6H), 1.27 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 207.7, 166.8, 100.7, 94.7, 81.1, 69.5, 60.8, 19.5, 19.4, 14.2. HRMS (ESI) calcd.for $C_{11}H_{14}O_2$ 179.1063 [M + H] $^{+}$, found. 179.1065.

23



4-(4-bromophenyl)oxazolidin-2-one (S7) In the first step, 2-amino-1-(4-bromophenyl) ethanol (300 mg, 1.39 mmol) was added to a dry round-bottomed flask, and then ultra-dry DCM (5 mL) and Et_3N(310 mg, 3.05 mmol). After stirring at room temperature for 10 min, triphosgene (110 mg, 5.55 mmol) was slowly added in portions, and stirred in an ice-water bath for 8 h. The reaction process was monitored

by thin-layer chromatography until the reaction was completed, extracted with 20 mL ×3 ethyl acetate, the organic phase was washed with saturated brine and dried over anhydrous sodium sulfate, and crude product of compound **S8** (310 mg) was obtained after distillation under reduced pressure, which was directly applied to the next step of reaction.

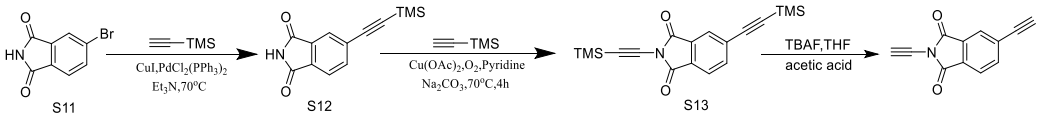
4-(4-bromophenyl)-3-((triisopropylsilyl)ethynyl)oxazolidin-2-one (S9) In the second step, the crude product of compound **S8** (200 mg) was added under the protection of N_2 anhydrous, then $CuSO_4$ (26.4 mg, 0.2 eq), K_3PO_4 (350.8 mg, 2.0eq), 1, 10-phenanthroline (74.4 mg, 0.5 eq) were added into the toluene(5 mL) which mixture was stirred at room temperature for 10 min. And then (2-bromoethynyl) triisopropylsilane (260.0 mg, 1.2 eq) was added dropwise to the system. The reaction mixture was stirred at reflux for 4 h. Monitor the reaction process with thin-layer chromatography until the reaction is complete, extracted with 20 mL× 3 ethyl acetate, washed the organic phase with saturated brine and dried over anhydrous sodium sulfate,. Fianlly we removed the solvent by distillation under reduced pressure and used PE: EA=7: 1 to purified by silica gel column chromatography to obtain compound **S9**(272.6 mg, yield 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, J = 8.5 Hz, 2H), 7.26 (d, J = 8.4 Hz, 2H), 5.06 (dd, J = 8.7, 7.4 Hz, 1H), 4.75 (t , J = 8.9 Hz, 1H), 4.25 (dd, J = 9.0, 7.4 Hz, 1H), 0.94 (d, J = 2.2 Hz, 21H).

3-((triisopropylsilyl)ethynyl)-4-(4-((triisopropylsilyl)ethynyl)phenyl)oxazolidin-2-one (S10) In the third step, compound **S9** (140.0 mg, 0.33 mmol), cuprous iodide (1.3 mg, 0.006 mmol), triphenylphosphine palladium dichloride (4.7 mg, 0.006 mmol) were added to anhydrous ethyl acetate(3 mL) under the protection of N_2, DIPEA (642.5 mg, 4.9 mmol) and triisopropylsilylacetylene (90.7 mg, 0.3 mmol) were added sequentially under stirring, stirred at room temperature for 10 min, and the reaction system was refluxed at 50°C for 6 h. The reaction process was monitored by thin-layer chromatography until the reaction was completed, extracted with 20 mL ×3 ethyl acetate, the organic phase was washed with saturated brine and dried over anhydrous sodium sulfate, and the solvent was distilled off under reduced pressure to obtain crude compound **S10** (160 mg), which was directly carried out next step of reaction.

3-ethynyl-4-(4-ethynylphenyl)oxazolidin-2-one(23) In the fourth step, crude compound **S10** (50 mg)was added anhydrous tetrahydrofuran(3 mL) under the protection of N_2, stired in an ice-water bath for 10 min, then added a solution of tetrabutylammonium fluoride in tetrahydrofuran (4 M, 57 μL), and continue stirring for 10 min in an ice-water bath. Monitor the reaction process with thin-layer

chromatography until the reaction is complete, extract with 10 mL× 3 ethyl acetate, washed the organic phase with saturated brine and dry over anhydrous sodium sulfate, and evaporated the solvent under reduced pressure. And PE:EA=3:1 was appied to purify the reaction production by silica gel column chromatography under the elution conditions , finally obtained compound **23** (32 mg, yield 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, J = 8.2 Hz, 2H), 7.37 – 7.33 (m, 2H), 5.12 – 5.07 (m, 1H), 4.80 – 4.75 (m, 1H), 4.28 – 4.23 (m, 1H), 3.17 (s, 1H), 2.76 (s, 1H). HRMS (ESI) calcd. for $C_{13}H_9NO_2$ 212.0633 $[M + H]^+$, found 212.0706.

24



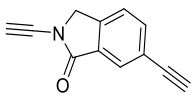
5-((trimethylsilyl)ethynyl)isoindoline-1,3-dione (S11) The first step, compound **S11** (904 mg, 4 mmol), $PdCl_2$ $(PPh_3)_2$ (56 mg, 0.08 mmol), CuI (30 mg, 0.16 mmol), Et_3N (860 uL, 6.2 mmol) were added in a dry round bottom flask, and N_2 Ultra-dry THF (20 mL) was added under protection, heated to 70°C, and then ethynyltrimethylsilane (706 uL, 5 mmol) was added slowly. The progress of the reaction was monitored by thin layer chromatography until the reaction completed. After cooling to room temperature, the mixture was diluted with 5 mL of ethyl acetate and filtered through celite. The filtrate was evaporated under reduced pressure, and the crude product was purified by silica gel column chromatography to obtain compound **S12** (900 mg, yield 92.6%)。^{1}H NMR (400 MHz, DMSO-*d6*) δ 11.50 (s, 1H), 7.88 – 7.79 (m, 3H), 0.27 (s, 9H). ^{13}C NMR (400 MHz, DMSO-*d6*) δ 168.98, 168.83, 137.77, 133.64, 132.69, 128.22, 125.98, 123.79, 103.84, 99.27, 90.14.

2,5-bis((trimethylsilyl)ethynyl)isoindoline-1,3-dione(S13) In the second step, compound **S12** (243 mg, 1 mmol), Na_2CO_3 (212 mg, 2 mmol), $Cu(OAc)_2$ (40 mg, 0.2 mmol), and 4 A molecular sieves (400 mg) were added to a dry round bottom flask. A solution of pyridine (161 uL, 2 mmol) in toluene (10 mL) was added to the reaction vial, which was then purged with the volume of 3-fold O_2. The reaction flask was stirred in an oil bath for 1 h (70°C.), and a solution of ethynyltrimethylsilane (216 uL, 1.2 mmol) was added to dry toluene (1 mL), and then slowly added to the reaction systems. The reaction mixture was stirred for 4 h. The reaction production was filtered through celite, concentrated under reduced pressure, and purified by silica gel column chromatography to obtain compound **S13** (100 mg, yield 29.5%). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 7.94 – 7.92 (m, 3H), 0.27 (s, 9H), 0.25 (s, 9H). ^{13}C NMR (400 MHz,

DMSO-*d6*) δ 164.91, 164.83, 138.57, 132.09, 130.93, 129.22, 126.98, 124.80, 103.37, 100.56, 86.60, 82.37, 0.33, 0.09.

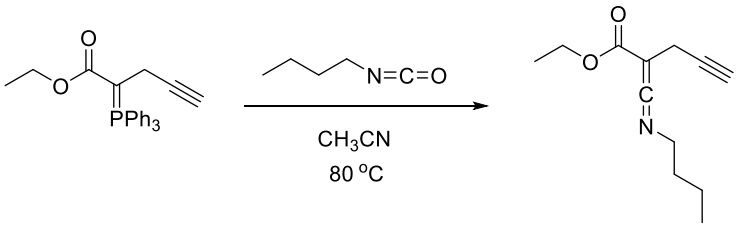
2,5-diethynylisoindoline-1,3-dione (24) In the third step, compound **S13** (50 mg, 0.147 mmol) was dissolved in 2 mL dry THF by adding a magnetic stirring bar in a single-neck round bottom flask and cooled in an ice bath to 4°C. Then acetic acid (16 uL, 0.294 mmol) was added to the reaction medium and stirred for 5 min. TBAF (1 M in THF) (48 uL, 0.354 mmol) was added dropwise to this stirred reaction medium over 30 min. The reaction continued to stir at 4°C until complete consumption of starting material and judged the reaction producs by thin layer chromatography analysis. The reaction was then returned to room temperature, then transferred to a separatory funnel, quenched with cold water, and extracted three times with dichloromethane. Dry over anhydrous sodium sulfate and evaporate under vacuum. And carried out silica gel column chromatography purification subsequently to obtain desired product **24** (17 mg，yield 59.3%)。^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.03 – 7.94 (m, 3H), 4.72 (s, 1H), 4.59 (s,1H). ^{13}C NMR (400 MHz, DMSO-*d6*) δ 165.18, 165.09, 138.86, 132.09, 131.09, 128.90, 127.12, 124.77, 86.36, 82.20, 70.92, 67.71. HRMS (ESI) calcd. for $C_{12}H_5NO_2$ 196.0320 $[M + H]^+$, found 196.0393.

25



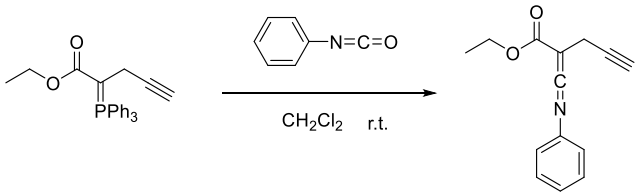
2,6-diethynylisoindolin-1-one (25) The synthetic route of **25** is similar to that of **24** (210 mg, yield 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, *J* = 7.9 Hz, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.59 (s, 1H), 4.73 (s, 2H), 3.39 – 3.30 (m, 1H), 3.13 (s, 1H), 1.07 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.38 (s), 140.81 (s), 132.73 (s), 129.47 (s), 127.29 (s), 126.63 (s), 124.65 (s), 82.43 (s), 80.69 (s), 73.63 (s), 62.36 (s), 51.82 (s). HRMS (ESI) calcd. for $C_{12}H_7NO$ 182.0600 $[M + H]^+$, found 182.0598.

26



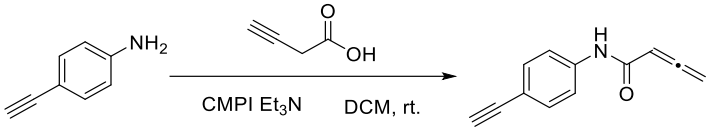
Ethyl 2-((butylimino)methylene)pent-4-ynoate (26):To a solution of phosphorane (2 mmol) in dry CH_3CN (10 mL) was added the *n*-butyl isocyanate (2 mmol) at r.t., then the mixture was stirred at 80 °C for 12 h, when the reaction was complete, the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc/petroleum ether, 1:10) to give product **26** yellow oil (0.286g, 69% yield); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 4.26-4.21 (m, 4H), 3.39 (t, J = 6.0 Hz, 1H), 3.32-3.26 (m, 2H), 2.82-2.80 (m, 2H), 1.53-1.47 (m, 2H), 1.38-1.33 (m, 2H), 1.29 (t, J = 8.0 Hz, 3H), 0.92 (t, J = 6.0 Hz, 3H); 13C NMR (CDCl3, 100 MHz) δ (ppm) 169.8, 166.4, 80.4, 70.4, 61.8, 51.6, 39.5, 31.4, 19.9, 19.0, 14.0, 13.6. HRMS (ESI) calcd.for $C_{12}H_{17}NO_2$ 208.1332 [M + H]$^+$, found. 208.1322.

27



Ethyl 2-((phenylimino)methylene)pent-4-ynoate (27) To a solution of phosphorane (2 mmol) in dry CH_2Cl_2 (10 mL) was added the phenyl isocyanate (2 mmol) at r.t., the mixture was stirred for 4-6 h, when the reaction was complete, the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc/petroleum ether, 1:6) to give product **27**.: yellow oil (0.332g, 73% yield); 1H NMR ($CDCl_3$, 400 MHz) δ (ppm) 7.43-7.32 (m, 5H), 4.25-4.18 (m, 4H), 3.21 (d, J = 2.8 Hz, 1H), 2.65-2.63 (m, 2H), 1.28-1.24 (m, 3H); 13C NMR ($CDCl_3$, 100 MHz) δ (ppm) 179.8, 172.2, 168.3, 137.5, 129.6, 128.4(minor), 124.3, 80.4, 80.3(minor), 70.4, 70.1(minor), 64.13, 61.0(minor), 60.8, 42.9, 19.8. HRMS (ESI) calcd.for $C_{14}H_{13}NO_2$ 228.1019 [M + H] $^+$, found.228.1013.

28

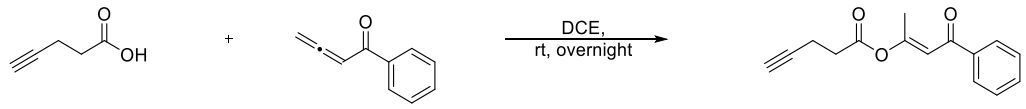


N-(4-ethynylphenyl)buta-2,3-dienamide(28) 3-butynoic acid (232 mmol, 2.76 mmol) and 2-chloro-1-methyl pyridinium iodide(CMPI) (1057 mg, 4.14 mmol) were charged in a 25 mL two neck oven dried RBF fitted with a magnetic stirrer bar. 8 mL dry dichloromethane was added to it and the mixture was

stirred under nitrogen for 1 h. A solution of 4-ethynylaniline (291 mg, 2.48 mmol) and triethylamine (376.43 mg, 3.72 mmol) in 6 mL dry dichloromethane was added to it drop wise after which the reaction mixture was stirred for another half an hour. After the work up as described above, product was purified by flash column chromatography (PE:EA=8:1) to get a light brown solid. Finally, it was readily isomerized to allenamide by treating with 1 eq. of triethylamine in dichloromethane(261 mg, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.50 (d, *J* = 8.3 Hz, 2H), 7.45 (d, *J* = 7.5 Hz, 2H), 5.76 (t, *J* = 6.7 Hz, 1H), 5.38 (d, *J* = 6.6 Hz, 2H), 3.05 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 207.24, 133.41, 128.23, 114.41, 87.07, 78.62, 76.82, 72.14. HRMS (ESI) calcd.for $C_{12}H_9NO$ 184.0757 $[M + H]^+$, found 184.0754.

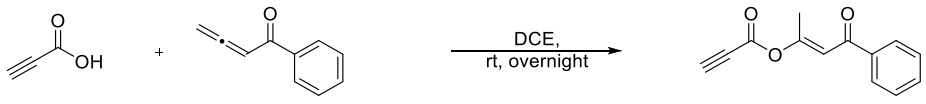
29-34 General Procedure: Alkynyl acid (0.2 mmol) and allenone (0.2 mmol) were dissolved in 4 mL of DCE. The mixture was stirred at rt overnight until no starting material monitoring by TLC. The reaction mixture was concentrated under vacuum and the residues purified by column chromatography on silica gel to afford the desired product.

29



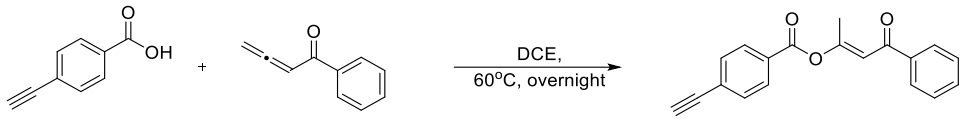
(E)-4-oxo-4-phenylbut-2-en-2-yl pent-4-ynoate (29) was synthesized by general procedure as a white solid (20.8 mg, 43 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 – 7.81 (m, 2H), 7.70 – 7.54 (m, 1H), 7.54 – 7.43 (m, 2H), 6.83 (d, *J* = 1.2 Hz, 1H), 2.75 (t, *J* = 7.0 Hz, 2H), 2.68 – 2.55 (m, 2H), 2.43 (d, *J* = 1.0 Hz, 3H), 2.06 (t, *J* = 2.6 Hz, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 190.3, 169.2, 163.5, 138.6, 132.9, 128.6, 128.2, 113.7, 81.8, 69.6, 33.6, 19.0, 14.4. HRMS (ESI) calcd. for $C_{15}H_{15}O_3$ $[M+H]^+$ 243.1021, found 243.1025.

30



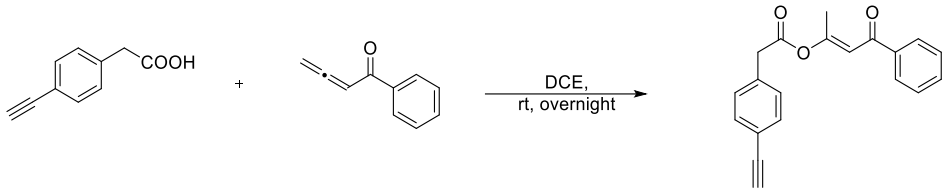
(E)-4-oxo-4-phenylbut-2-en-2-yl propiolate (30) was synthesized by general procedure as a white solid (21.7 mg, 51 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (dd, *J* = 7.2, 1.7 Hz, 2H), 7.59 (td, *J* = 7.3, 1.5 Hz, 1H), 7.55 – 7.42 (m, 2H), 6.93 – 6.84 (m, 1H), 3.10 (s, 1H), 2.45 (d, *J* = 0.8 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 190.0, 162.3, 149.6, 138.3, 133.2, 128.7, 128.2, 128.2, 114.4, 74.0, 18.6. HRMS (ESI) calcd. for $C_{13}H_{11}O_3$ $[M+H]^+$ 215.0703, found 215.0702.

31



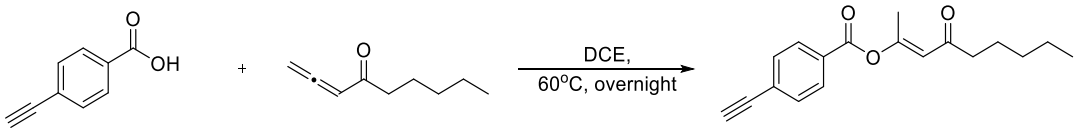
(E)-4-oxo-4-phenylbut-2-en-2-yl 4-ethynylbenzoate(31) was synthesized by general procedure as a white solid (49.9 mg, 86 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 – 8.05 (m, 2H), 8.02 – 7.93 (m, 2H), 7.66 – 7.61 (m, 2H), 7.61 – 7.54 (m, 1H), 7.49 (dd, *J* = 8.2, 6.9 Hz, 2H), 6.96 (d, *J* = 1.2 Hz, 1H), 3.32 (s, 1H), 2.55 (d, *J* = 1.0 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 190.3, 163.9, 163.4, 138.6, 133.0, 132.3, 130.0, 129.1, 128.6, 128.2, 127.8, 114.0, 82.6, 80.9, 19.1. HRMS (ESI) calcd. for $C_{19}H_{15}O_3$ $[M+H]^+$ 291.1021, found 291.1017.

32



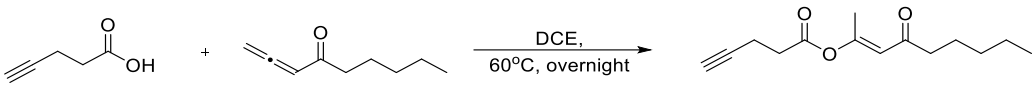
(E)-4-oxo-4-phenylbut-2-en-2-yl2-(4-ethynylphenyl)acetate(32)was synthesized by general procedure as a white solid (44.4 mg, 73 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 – 7.87 (m, 2H), 7.61 – 7.44 (m, 5H), 7.37 – 7.30 (m, 2H), 6.79 (d, *J* = 1.1 Hz, 1H), 3.81 (s, 2H), 3.12 (s, 1H), 2.38 (d, *J* = 1.0 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 190.3, 168.5, 168.1, 163.5, 138.5, 134.0, 132.9, 132.6, 132.4, 129.8, 129.3, 128.6, 128.2, 121.5, 113.7, 83.4, 77.7, 41.4, 18.8. HRMS (ESI) calcd. for $C_{20}H_{17}O_3$ $[M+H]^+$ 305.1178, found 305.1176.

33



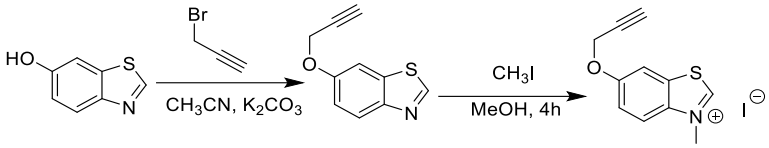
(E)-4-oxonon-2-en-2-yl 4-ethynylbenzoate(33) was synthesized by general procedure as a white solid (47.8 mg, 84 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 – 7.98 (m, 2H), 7.72 – 7.52 (m, 2H), 6.23 (d, *J* = 1.2 Hz, 1H), 3.30 (s, 1H), 2.55 – 2.40 (m, 5H), 1.65 (dd, *J* = 14.7, 7.4 Hz, 3H), 1.41 – 1.29 (m, 5H), 0.92 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 200.1, 163.4, 162.5, 132.3, 129.9, 129.9, 116.3, 82.6, 80.8, 45.0, 31.4, 23.7, 22.5, 18.7, 13.9. HRMS (ESI) calcd. for $C_{18}H_{21}O_3$ $[M+H]^+$ 285.1491, found 285.1489.

34



(E)-4-oxonon-2-en-2-yl pent-4-ynoate (34) was synthesized by general procedure as a white solid (41.1 mg, 87 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.09 (d, *J* = 1.1 Hz, 1H), 2.69 (t, *J* = 7.0 Hz, 2H), 2.57 (td, *J* = 8.0, 7.4, 1.8 Hz, 2H), 2.46 (t, *J* = 7.4 Hz, 2H), 2.34 (d, *J* = 1.0 Hz, 3H), 2.04 (t, *J* = 2.6 Hz, 1H), 1.68 – 1.55 (m, 3H), 1.31 (dtd, *J* = 11.7, 6.8, 4.0 Hz, 5H), 0.90 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 200.1, 169.2, 168.7, 162.1, 116.1, 82.3, 81.8, 69.6, 69.2, 44.9, 33.5, 31.4, 31.3, 23.7, 22.5, 18.6, 14.3, 14.1, 13.9, 13.9. HRMS (ESI) calcd. for $C_{14}H_{21}O_3$ $[M+H]^+$ 237.1491, found 237.1492.

35

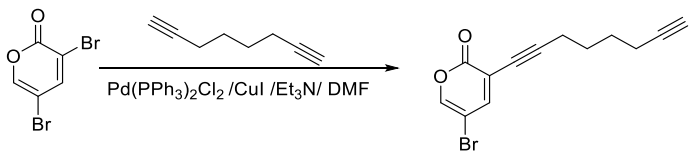


S14

6-(prop-2-yn-1-yloxy)benzo[d]thiazole(S14): To a stirred solution of benzo[d]thiazol-6-ol (1.0 eq) in MeCN was added 3-bromoprop-1-yne (1.5 eq) and potassium carbonate (2 eq). The resulting mixture was stirred overnight. After the reaction was completed, the solution was concentrated under reduced pressure, and the reaction production was purified by flash column chromatography to get compound. **S14**.

3-methyl-6-(prop-2-yn-1-yloxy)benzo[d]thiazol-3-ium iodide (35): To a stirred solution of **S14**(1eq) in methanol systems was added iodomethane (1.5 eq). The resulting mixture was stirred for 4 h. After the reaction was completed, the solution was concentrated under reduced pressure, and the residue was washed with EA and DCM to produce pure **35** (320 mg, yield 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (s, 1H), 8.24 (d, *J* = 9.3 Hz, 1H), 8.10 (d, *J* = 2.5 Hz, 1H), 7.59 (dd, *J* = 9.3, 2.5 Hz, 1H), 4.99 (d, *J* = 2.4 Hz, 2H), 4.36 (s, 3H), 3.68 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.23, 157.53, 136.39, 133.42, 119.80, 118.67, 108.85, 79.65, 78.78, 56.92.

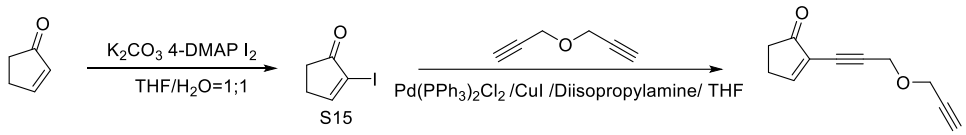
36



5-bromo-3-(octa-1,7-diyn-1-yl)-2H-pyran-2-one (36) To a mixture of 580 mg of 3,5-dibromo- 2-pyrone (2.28 mmol), 90 mg of $Pd(PPh_3)_2Cl_2$ (0.11 mmol), and CuI (44 mg, 0.24 mmol) in 1,4-dioxane (30 mL),

Then mixture was added Et_3N (0.38 mL, 2.74 mmol) and octa-1,7-diyne (290 mg , 2.74 mmol). After stirring for 0.5 h under N_2, the reaction mixture was filtered through a plug of Celite and the filtered material was washed with ether. The filtrate was washed with H_2O, dried over $MgSO_4$, concentrated, and chromatographed (PE/EA=10:1) to obtain **36** (820 mg, 83% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.55 (d, *J* = 2.5 Hz, 1H), 7.45 (d, *J* = 2.6 Hz, 1H), 2.51 (t, *J* = 6.7 Hz, 2H), 2.26 (td, *J* = 6.7, 2.7 Hz, 2H), 1.98 (t, *J* = 2.6 Hz, 1H), 1.78 – 1.67 (m, 4H).^{13}C NMR (101 MHz, $CDCl_3$) δ 159.16, 148.22, 146.53, 115.03, 100.46, 99.62, 83.94, 74.03, 68.73, 27.49, 27.13, 19.32, 17.96. HRMS (ESI) calcd. for $C_{13}H_{11}BrO_2$ 279.0015 $[M+H]^+$, found 279.0007.

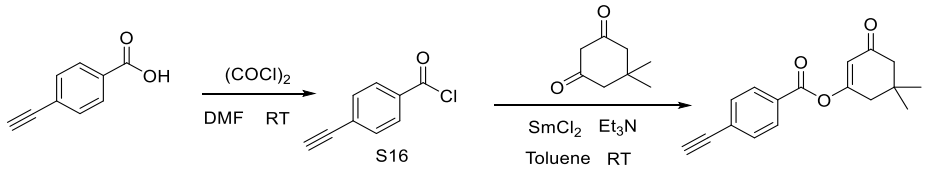
37



2-iodocyclopent-2-en-1-one (S15) Cyclopent-2-en-1-one (1eq) was added to the system THF:H_2O=1:1, then K_2CO_3 (2eq) added. Followed by ,the compounds DMAP (1eq) and I_2 (1.5eq) were added the mixture reaction systems . The mixed system was stirred for 1 h at ambient temperature, and then **S15** was obtained directly for the next step of the reaction without silica gel column purification.

2-(3-(prop-2-yn-1-yloxy)prop-1-yn-1-yl)cyclopent-2-en-1-one (37) To a mixture of 2-iodocyclopent-2-en-1-one (207 mg , 1 mmol), $Pd(PPh_3)_2Cl_2$ (90 mg , 0.11 mmol), and CuI (44 mg , 0.24 mmol) in THF (30 mL). Then miture was added Diisopropylamine(1 mL) and 3-(prop-2-yn-1-yloxy)prop-1-yne (257 mg , 2.74 mmol). After stirring for 2 h under N_2 atmosphere, the reaction mixture was filtered through a plug of Celite. The filtrate was washed with H_2O, then dried with $MgSO_4$, concentrated and chromatographed (PE /EA=5:1) to gain **37** (348 mg, yield 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (t, *J* = 3.0 Hz, 1H), 4.47 (s, 2H), 4.29 (t, *J* = 4.1 Hz, 2H), 2.82 – 2.67 (m, 2H), 2.53 – 2.45 (m, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 205.58 (s), 166.00 (s), 129.49 (s), 91.00 (s), 78.83 (s), 77.53 (s), 75.15 (s), 57.21 (s), 56.67 (s), 33.93 (s), 27.40 (s). HRMS (ESI) calcd. for $C_{11}H_{10}O_2$ 175.0754 $[M+H]^+$found 175.0755.

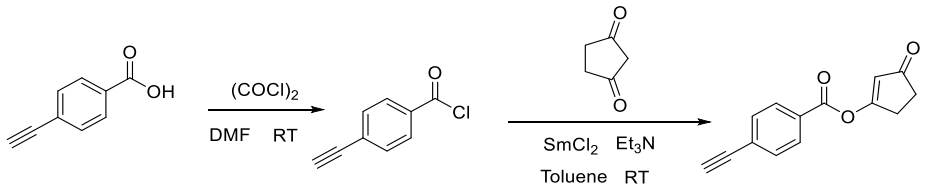
38



4-ethynylbenzoyl chloride (S16) Under the protection of argon, 4-ethynylbenzoyl chloride (**S16**)., 4-ethynylbenzoic acid (293 mg, 2.0 mmol) were dissolved in ultra-dry DCM solvent, and then oxalyl chloride (514.9 μL, 6.0 mmol) was added dropwise. The mixture was stirred overnight at room temperature, and the solvent was removed under reduced pressure. The obtained crude product was added to anhydrous Na_2SO_4 dried DCM to remove excessive oxalyl chloride to obtain a red solid S1, which could be directly used for the next reaction without purification.

5,5-dimethyl-3-oxocyclohex-1-en-1-yl 4-ethynylbenzoate (38). **S16** (328 mg, 2.0 mmol) and 5,5-dimethylcyclohexane-1,3-dione (255 mg, 1.82 mmol), $SmCl_3$ (25.6 mg, 0.1 mmol), Et_3N (303.6 μL, 2.18 mmol) were dissolved in toluene solution (20 mL) under argon protection. The reaction was placed at room temperature for 4 h. The reaction mixture was quenched with water and extracted three times with ethyl acetate (EA). The combined organic layer was washed with salt water, dried with anhydrous Na_2SO_4, and concentrated in vacuum. The residue was purified by silica gel column chromatography (eluting with EA:PE=1:6) to obtain a yellow solid (410.3 mg, yield 84.1 %).^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.05 – 8.00 (m, 2H), 7.62 – 7.57 (m, 2H), 6.04 (t, *J* = 1.3 Hz, 1H), 3.29 (s, 1H), 2.54 (d, *J* = 1.4 Hz, 2H), 2.32 (s, 2H), 1.15 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 199.11, 168.91, 162.72, 132.71, 130.56, 127.95, 116.66, 85.19, 82.92, 50.70, 41.69, 33.36, 28.05. HRMS (ESI) calcd.for $C_{17}H_{16}O_3$ 291.0992 $[M + H]^+$, found 291.0981.

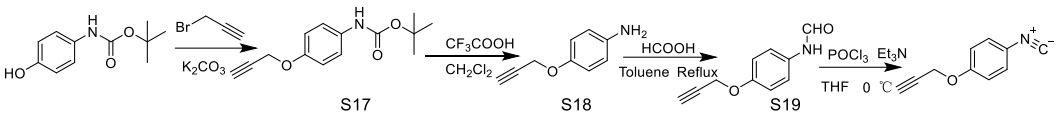
39



3-oxocyclopent-1-en-1-yl 4-ethynylbenzoate(**39**). S16 (328 mg, 2.0 mmol) and cyclopentane-1,3-dione (180 mg, 1.82 mmol), $SmCl_3$ (25.6 mg, 0.1 mmol), Et_3N (303.6 μL, 2.18 mmol) were dissolved in toluene solution (20 mL) under argon protection. The reaction was placed at room temperature for 4 h. The reaction mixture was quenched with water and extracted three times with ethyl acetate (EA). The combined organic layer was washed with salt water, dried with anhydrous Na_2SO_4, and concentrated in vacuum. The residue was purified by silica gel column chromatography (eluting with EA: DCM =1:60) to obtain a white solid (89mg, yield 19.7 %).^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.13 – 8.07 (m, 2H),

7.67 – 7.59 (m, 2H), 6.41 (d, *J* = 1.7 Hz, 1H), 2.96 – 2.91 (m, 2H), 2.58 – 2.53 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 201.84, 174.87, 156.78, 127.75, 125.50, 123.72, 123.24, 112.27, 77.63, 76.65, 28.76, 24.07. HRMS (ESI) calcd.for $C_{14}H_{10}O_3$ 249.0522 $[M + H]^+$, found 249.0518.

40



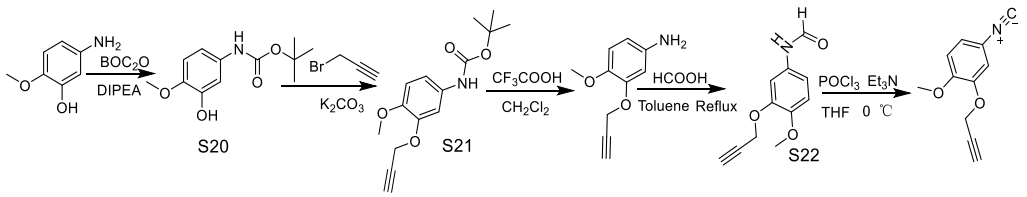
tert-butyl (4-(prop-2-yn-1-yloxy)phenyl)carbamate(S17) Synthesis of **40** was carried out similar to literature procedure [29](**S17**). a 100 mL round-bottom flask was added tert-butyl (4-hydroxyphenyl) carbamate (1.045 g, 5 mmol), 3-bromopropyne (900 mg, 7.5 mmol), potassium carbonate (1.38 g, 10 mmol) and 15 mL DMF. The reaction mixture was stirred at room temperature for 2 h, and then was quenched by addition of water and extracted with ethyl acetate 2-3 times. The organic phase was washed twice with saturated brine, and dried over anhydrous Na_2SO_4. The organic solvent was evaporated under reduced pressure, the residue was purified by silica gel column (PE:EA=8:1) to afford **S17**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.18 (s, 1H), 7.36 (d, *J* = 8.5 Hz, 2H), 7.03 – 6.65 (m, 2H), 4.72 (d, *J* = 2.4 Hz, 2H), 3.54 (s, 1H), 1.47 (s, 9H).

4-(prop-2-yn-1-yloxy)aniline (S18) a 50 mL round bottom flask was added **S17** (1 g, 4 mmol), 4 mL CH_2Cl_2 and 4 mL trifluoroacetic acid. After later, the extra CH_2Cl_2 and TFA were evaporated under reduced pressure. then, 100 mL saturated sodium bicarbonate was added to neutralize the extra acid and extracted with ethyl acetate, the organic phase was washed twice with saturated brine, and dried over anhydrous Na_2SO_4. Upon evaporation of the solvent in vacuo and the crude product was purified by flash column (PE:EA=3:1) to afford **S18** (500 mg, 84%). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 6.80 – 6.65 (m, 2H), 6.59 – 6.45 (m, 2H), 4.69 (s, 2H), 4.61 (s, 2H), 3.48 (t, J = 2.4 Hz, 1H).

N-(4-(prop-2-yn-1-yloxy)phenyl)formamide (S19) A 50 mL two round bottom flask was added **S18** (441 mg, 3 mmol), 8 mL toluene and formic acid (230 μL, 6 mmol). The reaction mixture was stirred at 110℃ for 1 h. Then the mixture was evaporated under reduced pressure and the crude product was purified by flash column (PE:EA=2:1) to afford **S19** (420 mg, 80%). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.08 (s, 1H), 8.62 (d, J = 11.1 Hz, 1H), 8.22 (d, J = 2.0 Hz, 1H), 7.60 – 7.47 (m, 2H), 7.14 (d, J = 9.0 Hz, 1H), 7.01 – 6.87 (m, 3H), 4.76 (d, J = 2.4 Hz, 2H), 3.56 (t, J = 2.4 Hz, 1H).

1-isocyano-4-(prop-2-yn-1-yloxy)benzene (40) a 100 mL two round bottom flask was added **S19** (525 mg, 3 mmol), 6 mL CH_2Cl_2 and triethylamine (1.25 mL, 9 mmol), the reaction mixture was stirred in ice bath and $POCl_3$ (335 μL, 3.6 mmol) was added slowly. After stirring for 1 h in room temperature, the reaction mixture was cooled to 0 ℃, then poured to 20 mL saturated sodium bicarbonate solution and stirred for 30 min in 0 ℃ to neutralize the extra acid. The mixture was extracted with ethyl acetate, washed with brine and dried over by anhydrous Na_2SO_4. evaporated the reaction solvent in vacuo, the crude product was purified by flash column (PE:EA=15:1) to afford **40** (400 mg, 90%).^{1}H NMR (400 MHz, DMSO-d_6) δ 7.57 – 7.47 (m, 2H), 7.15 – 6.99 (m, 2H), 4.88 (d, J = 2.4 Hz, 2H), 3.62 (t, J = 2.4 Hz, 1H). ^{13}C NMR (151 MHz, DMSO) δ 163.39, 158.07, 128.30, 119.60, 116.34, 79.22, 56.30. HRMS (ESI) calcd. for $C_{10}H_7NO$ 158.0600, $[M + H]^+$, found 158.0603.

41



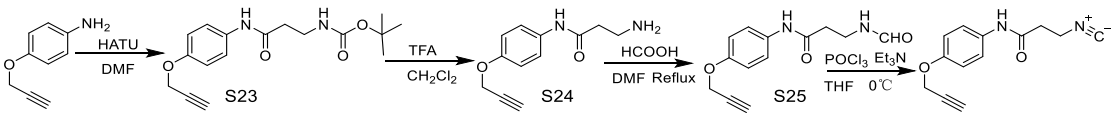
tert-butyl (3-hydroxy-4-methoxyphenyl)carbamate (S20) To a 100 mL round-bottom flask was added 5-amino-2-methoxyphenol (1.39 g, 10 mmol), di-tert-butyl dicarbonate (4.5 mL, 20 mmol), DIPEA (5 mL, 30 mmol) and 15 mL THF. The reaction mixture was stirred at 60℃ for 1 h, quenched by addition of water and extracted with ethyl acetate 2-3 times. The organic phase was washed twice with saturated brine, and dried over anhydrous Na_2SO_4. Upon evaporation of the solvent in vacuo, the crude product was purified by silica gel column (PE:EA=6:1) to afford **S20** (1.4 g, 60%). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.03 (s, 1H), 8.94 (s, 1H), 7.01 (s, 1H), 6.78 (d, J = 2.6 Hz, 2H), 3.69 (s, 3H), 1.45 (s, 9H).

tert-butyl (4-methoxy-3-(prop-2-yn-1-yloxy)phenyl)carbamate (S21) To a 100 mL round-bottom flask was added **S20** (1.2 g, 5 mmol), 3-bromopropyne (900 mg, 7.5 mmol), potassium carbonate (1.38 g, 10 mmol) and 15 mL DMF. The reaction mixture was stirred at 60℃ for 1 h, quenched by addition of water and extracted with ethyl acetate 2-3 times. The organic phase was washed twice with saturated brine, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was purified by silica gel column to afford **S21** (1.24 g, 90%).^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.17 (s, 1H), 7.26 – 7.19 (m, 1H), 7.00 (d, J = 8.8 Hz, 1H), 6.87 (d, J = 8.8 Hz, 1H), 4.69 (d, J = 2.4 Hz, 2H), 3.73 (s, 3H), 3.56 (t, J = 2.4 Hz, 1H), 1.46 (s, 9H).

N-(4-methoxy-3-(prop-2-yn-1-yloxy)phenyl)formamide (S22) To a 50 mL round bottom flask was added **S21** (1.38 g, 5 mmol), 5 mL CH_2Cl_2 and 5 mL trifluoroacetic acid, after later, the extra CH_2Cl_2 and TFA were evaporated under reduced pressure. Then 100 mL saturated sodium bicarbonate was added to neutralize the remaining acid and extracted with ethyl acetate, the organic phase was washed twice with saturated brine, and dried over anhydrous Na_2SO_4. Upon evaporation of the solvent in vacuo, the crude product was purified by flash column (PE:EA=3:1) to afford the compound (708 mg, 80%). The reaction product was added to two round bottom flask (50 mL) with 10 mL toluene and formic acid (377 μL, 10 mmol) and the reaction was stirred at 110 ℃ for 1 h. The mixture was evaporated under reduced pressure and the crude product was purified by flash column(PE:EA=2:1) to afford **S22** (492 mg, yield 60%).^{1}H NMR (400 MHz, DMSO-d_6) δ 10.02 (d, *J* = 34.7 Hz, 1H), 8.63 (d, *J* = 11.0 Hz, 1H), 8.20 (d, *J* = 2.0 Hz, 1H), 7.34 (d, *J* = 2.4 Hz, 1H), 7.19 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.94 (d, *J* = 8.9 Hz, 1H), 6.74 (dd, *J* = 8.6, 2.5 Hz, 1H), 4.76 (dd, *J* = 19.0, 2.4 Hz, 2H), 3.73 (d, *J* = 1.7 Hz, 3H), 3.57 (t, *J* = 2.5 Hz, 1H).

4-isocyano-1-methoxy-2-(prop-2-yn-1-yloxy)benzene (41) To a 100 mL two round bottom flask was added **S22**(308 mg, 1.5 mmol), 4 mL CH_2Cl_2 and triethylamine (0.7 mL, 4.5 mmol). After that, the reaction mixture was stirred in ice bath and $POCl_3$ (118 μL, 1.8 mmol) was added slowly. After stirring for 1 h in room temperature, the reaction mixture was cooled to 0℃ then poured to 20 mL saturated sodium bicarbonate solution and stirred for 30 min in 0℃ to neutralize the remaining acid. The mixture was extracted with ethyl acetate and washed with brine. Upon evaporation of the solvent in vacuo, the crude product was purified by flash column (PE:EA=10:1) to afford **41** (200 mg, yield 71 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.25 (d, *J* = 2.4 Hz, 1H), 7.19 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.05 (d, *J* = 8.6 Hz, 1H), 4.86 (d, *J* = 2.4 Hz, 2H), 3.81 (s, 3H), 3.64 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.91, 150.58, 146.94, 120.49, 118.51, 112.57, 112.17, 79.33, 79.09, 56.68, 56.30 HRMS (ESI) calcd. for $C_{11}H_9NO_2$ 188.0706 $[M + H]^+$, found 188.0703.

42



tert-butyl (3-oxo-3-((4-(prop-2-yn-1-yloxy)phenyl)amino)propyl)carbamate (S23) To a 100 mL round-bottom flask was added 4-(prop-2-yn-1-yloxy)aniline (1.47 g, 10 mmol), HATU (4.182 g, 11 mmol) and 20 mL DMF, the mixture stirred for 5 min. then DIPEA (3.5 mL, 20 mmol) and 3-((tert-

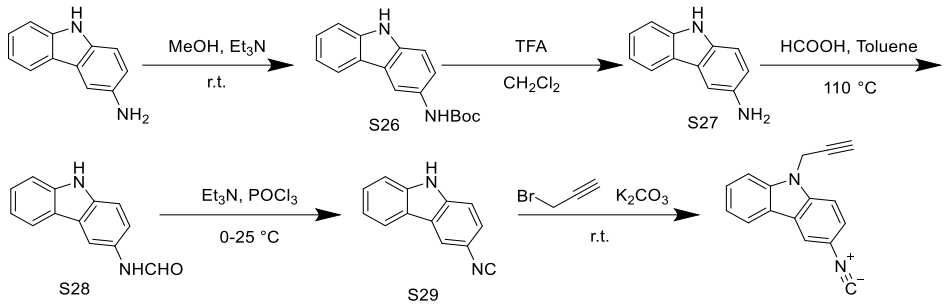
butoxycarbonyl)amino)propanoic acid (2.08 g, 11 mmol) was added. The reaction was stirred at room temperature for 2 h, quenched by addition of water and extracted with ethyl acetate 2-3 times. The organic phase was washed twice by saturated sodium chloride, dried by anhydrous Na_2SO_4 and purified by flash column to afford **S23** (2.87 g, yield 90%).^{1}H NMR (400 MHz, DMSO-d_6) δ 9.85 (s, 1H), 7.50 (d, *J* = 9.0 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 3H), 4.74 (d, *J* = 2.4 Hz, 2H), 3.57 (t, *J* = 2.4 Hz, 1H), 3.23 – 3.17 (m, 2H), 2.43 (t, *J* = 7.2 Hz, 2H), 1.38 (s, 9H).

3-amino-N-(4-(prop-2-yn-1-yloxy)phenyl)propanamide (S24) To a 50 mL round bottom flask was added **S23** (0.954 g, 3 mmol), CH_2Cl_2 (5 mL)and trifluoroacetic acid(5 mL). The reaction was stirred for 0.5 h, then was evaporated under reduced pressure and the crude product was purified by flash column (DCM:MeOH=20:1) to afford **S24** (410 mg, yield 63 %).^{1}H NMR (400 MHz, DMSO-d_6) δ 10.10 (s, 1H), 7.82 (s, 3H), 7.51 (d, *J* = 9.1 Hz, 2H), 6.94 (d, *J* = 9.0 Hz, 2H), 4.75 (d, *J* = 2.4 Hz, 2H), 3.58 (t, *J* = 2.3 Hz, 1H), 3.08 (t, *J* = 6.8 Hz, 2H), 2.67 (t, *J* = 6.8 Hz, 2H).

3-formamido-N-(4-(prop-2-yn-1-yloxy)phenyl)propanamide (S25) To a 50 mL round bottom flask was added **S24** (654 mg, 3 mmol), 10 mL DMF and 3 mL formic acid. The mixture was stirred at 110 ℃ for 3 h, then was evaporated under reduced pressure and the crude product was purified by flash column (DCM:MeOH=5:1) to afford **S25** (221 mg, yield 30 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.88 (s, 1H), 8.15 (d, *J* = 6.0 Hz, 1H), 8.02 – 7.98 (m, 1H), 7.57 – 7.47 (m, 2H), 6.92 (d, *J* = 9.0 Hz, 2H), 4.75 (d, *J* = 2.4 Hz, 2H), 3.56 (d, *J* = 2.4 Hz, 1H), 3.36 (d, *J* = 7.6 Hz, 2H), 2.47 (d, *J* = 6.6 Hz, 2H).

2-isocyano-N-(4-(prop-2-yn-1-yloxy)phenyl)propanamide (42) To a 50 mL two round bottom flask was added **S25** (147.6 mg, 0.6 mmol), THF (3 mL) and triethylamine (0.24 mL, 1.8 mmol), the resulting mixture was stirred in ice bath and $POCl_3$ (66 μL, 0.7 mmol) was added slowly. After stirring for 1 h in room temperature, the reaction mixture was cooled to 0℃ then poured to 20 mL saturated sodium bicarbonate solution and stirred for 30 min in 0℃ to neutralize the remaining acid. The mixture was extracted with ethyl acetate, washed with brine and dried by anhydrous Na_2SO_4. Upon evaporation of the solvent in vacuo, the crude product was purified by flash column (PE:EA=2:1) to afford **42** (103 mg, yield 76 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.00 (s, 1H), 7.51 (d, *J* = 9.1 Hz, 2H), 6.95 (d, *J* = 9.0 Hz, 2H), 4.75 (d, *J* = 2.4 Hz, 2H), 3.75 (d, *J* = 5.3 Hz, 2H), 3.54 (t, *J* = 2.4 Hz, 1H), 2.70 (dt, *J* = 6.8, 3.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 167.54, 156.27, 153.58, 133.14, 120.97, 115.46, 79.83, 78.63, 55.98, 38.13, 36.00. HRMS (ESI) calcd. for $C_{26}H_{19}N_3O_4S$ 228.0899 $[M + H]^+$, found 229.0972.

43



Tert-butyl (9H-carbazol-3-yl)carbamate (**S26**). Add Boc_2O (598.86mg, 2.74mmol) slowly to a solution of 9H-carbazol-3-amine(500 mg, 2.74 mmol) and triethylamine (286 μL, 2.06 mmol) in methanol (10 mL). Stir the reaction mixture at room temperature for 24 h. Remove the solvent by rotary evaporation and added water to the residue. Collect the precipitate by filtration. Wash the precipitate with water and dry under vacuum to obtain **S26** (774 mg, 99%) as a white solid, which can be directly used for the next reaction without purification.

9H-carbazol-3-amine (**S27**). To a 50 mL round bottom flask was added **S26** (700 mg, 2.48 mmol), 4 mL CH_2Cl_2 and 4 mL trifluoroacetic acid. A few minutes later, the extra CH_2Cl_2 and TFA were evaporated under reduced pressure to obtain **S27**(450 mg, 99%), which can be directly used for the next reaction without purification.

N-(9H-carbazol-3-yl) formamide (**S28**). To a 50 mL two round bottom flask was added **S27**(400 mg, 2.2 mmol), 8 mL toluene and formic acid (166 μL, 4.4 mmol). The reaction mixture was stirred at 110℃ for 1 h, then the mixture was evaporated under reduced pressure and then poured to 20 mL saturated sodium bicarbonate solution to neutralize the remaining acid. The mixture was extracted with dichloromethane and washed with brine. Upon evaporation of the solvent in vacuo to obtain **S28**(450 mg, 97%), the crude product can be directly used for the next reaction without purification.

3-isocyano-9H-carbazole (**S29**). To a 50 mL two round bottom flask was added **S28** (400 mg, 1.9 mmol), 6 mL CH_2Cl_2 and triethylamine (794 μL, 5.7 mmol), the reaction mixture was stirred in ice bath and $POCl_3$ (212.5 μL, 2.28 mmol) was added slowly. After stirring for 1 h in room temperature, the reaction mixture was cooled to 0 ℃ then poured to 20 mL saturated sodium bicarbonate solution and stirred for 30 min in 0℃ to neutralize the remaining acid. The mixture was extracted with ethyl acetate and washed with brine. Upon evaporation of the solvent in vacuo to obtain **S29** (346 mg, 95%), the crude product can be directly used for the next reaction without purification.

N-methylidyne-9-(prop-2-yn-1-yl)-9H-carbazol-3-aminium (**43**). To a 50 mL two round bottom flask was added **S29** (307 mg, 1.56 mmol), 3-bromopropyne (202 μL, 2.34 mmol), potassium carbonate (431 mg, 3.12 mmol) and 15 mL DMF. Upon evaporation of the solvent in vacuo, the crude product was purified by flash column (PE:EA=100:1) to afford **43**(306 mg, yield 85 %).^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.48 (d, J = 2.0 Hz, 1H), 8.25 (dt, J = 7.9, 1.0 Hz, 1H), 7.78 (d, J = 8.7 Hz, 1H), 7.74 (d, J = 8.3 Hz, 1H), 7.66 (dd, J = 8.7, 2.0 Hz, 1H), 7.57 (m, J = 8.3, 7.1, 1.2 Hz, 1H), 7.33 – 7.28 (m, 1H), 5.37 (d, J = 2.5 Hz, 2H), 3.29 (t, J = 2.4 Hz, 1H).^{13}C NMR (151 MHz, DMSO-*d6*) δ 140.87, 139.58, 127.57, 124.35, 123.11, 122.19, 121.58, 120.74, 119.41, 111.05, 110.61, 79.14, 75.38, 32.58. HRMS (ESI) calcd.for $C_{16}H_{10}N_2$ 231.0917 $[M + H]^+$, found 231.0919.

6. Reference

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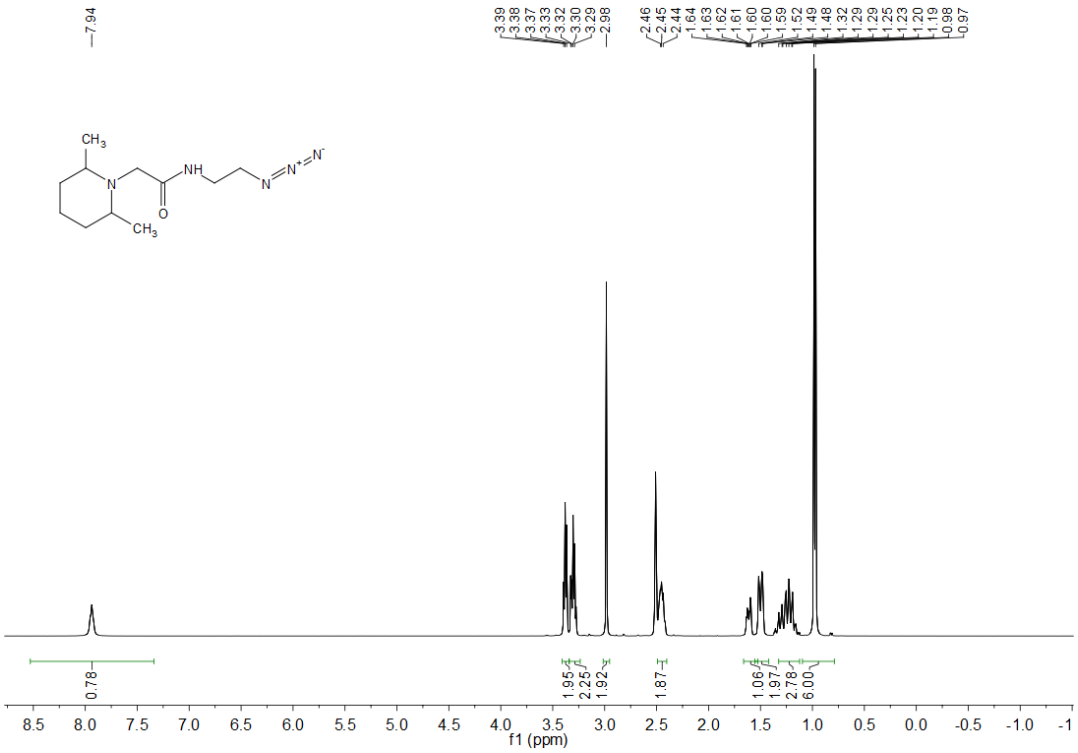
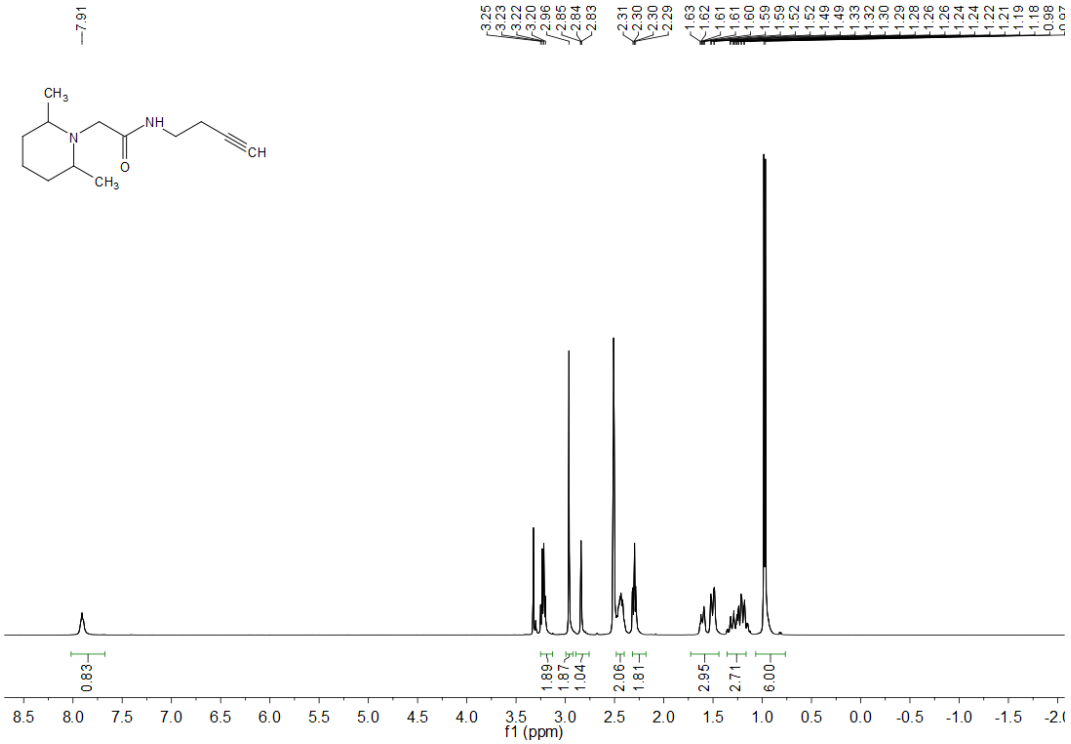
12 Chen, Y., et al., Addition of Isocyanide-Containing Amino Acids to the Genetic Code for Protein Labeling and Activation. *ACS Chem Biol,* 2019. **14**(12): p. 2793-2799.

7. NMR spectra

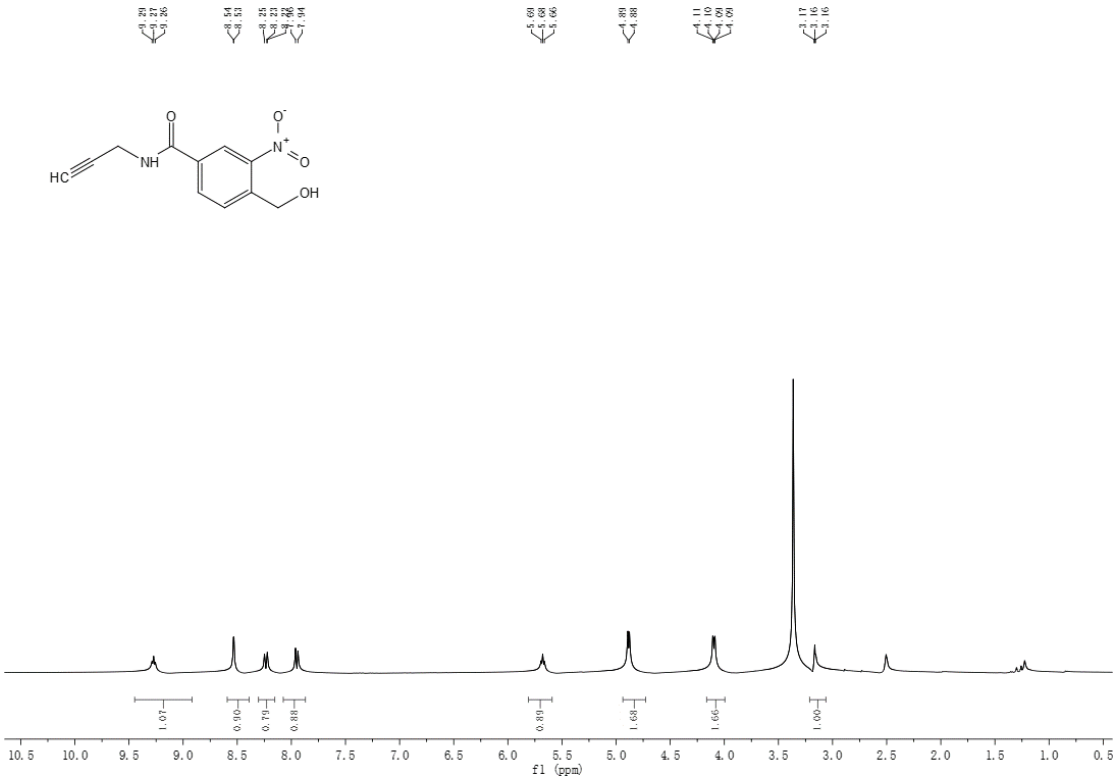
DMP tag1 ^{1}H NMR



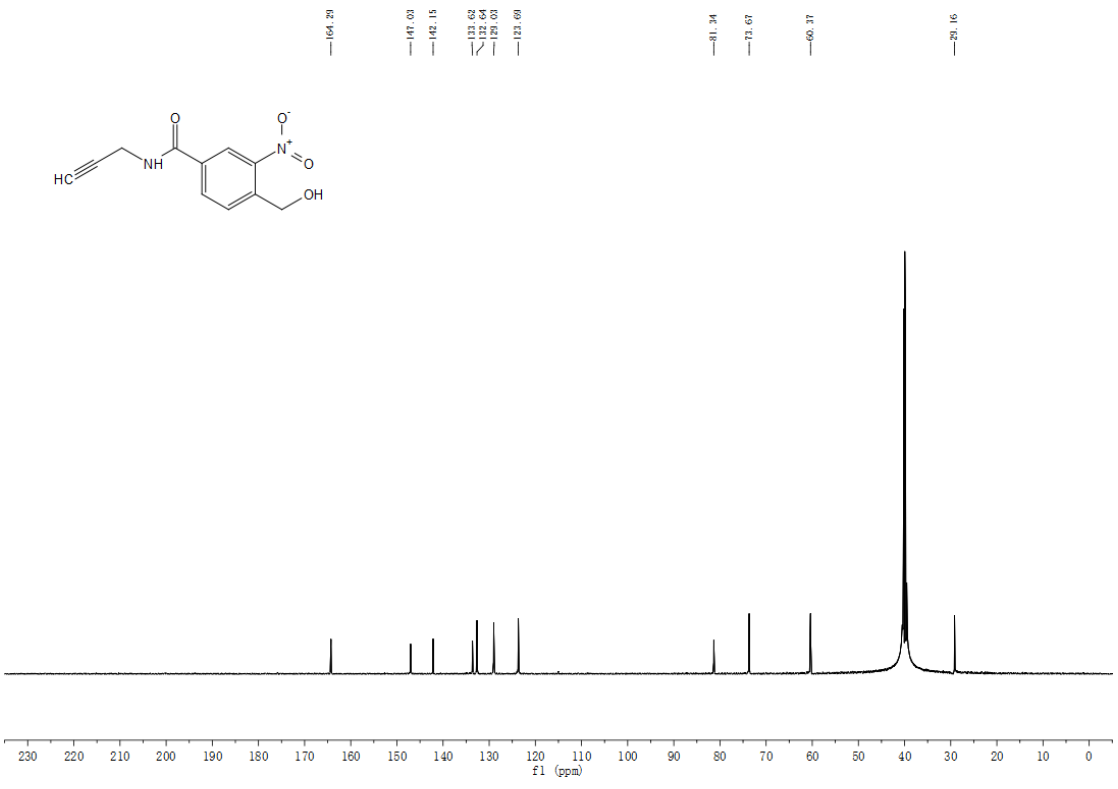
DMP tag2 ^{1}H NMR



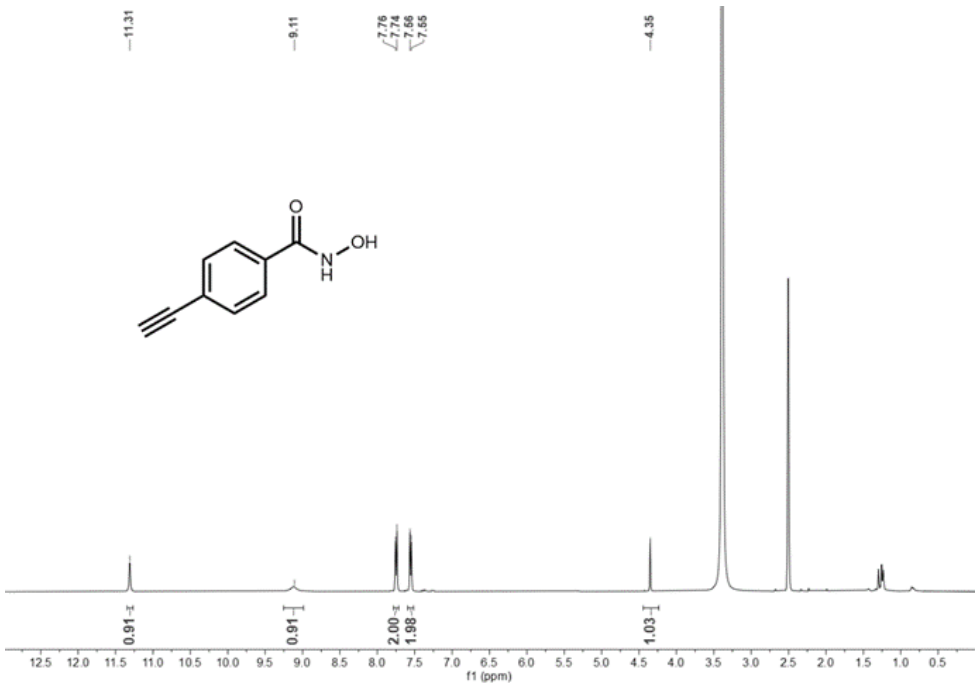
2 ^{1}H NMR



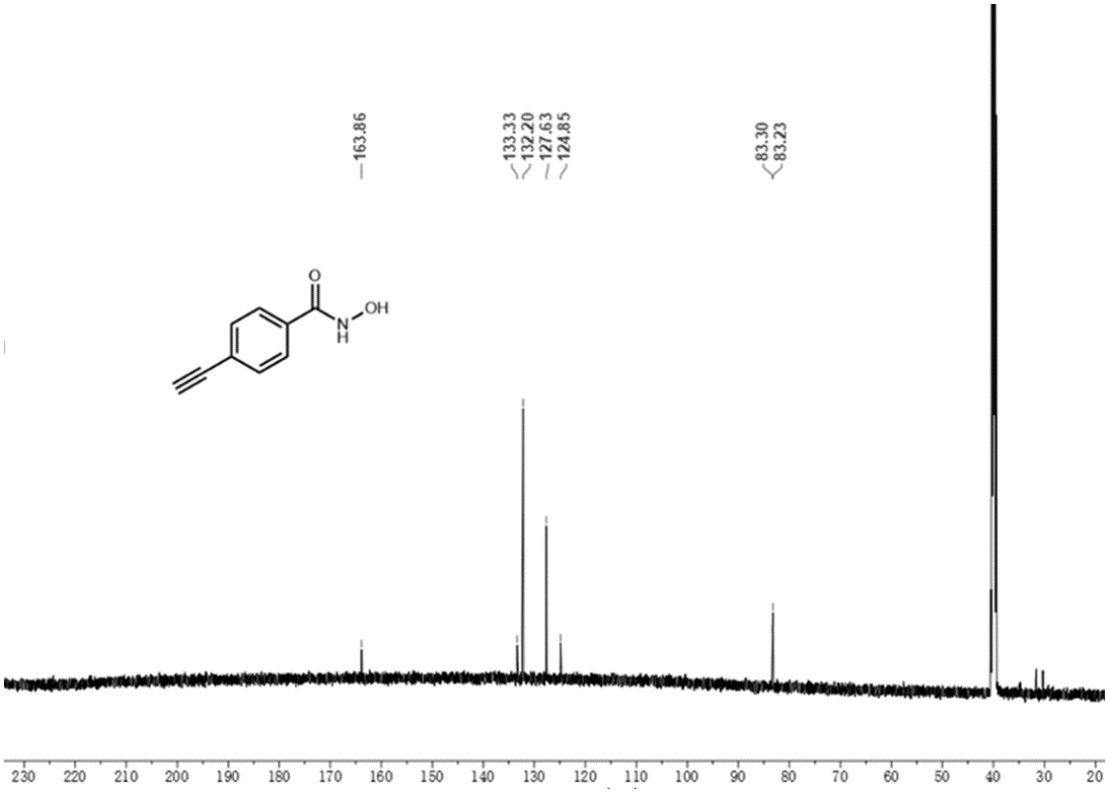
2 ^{13}C NMR



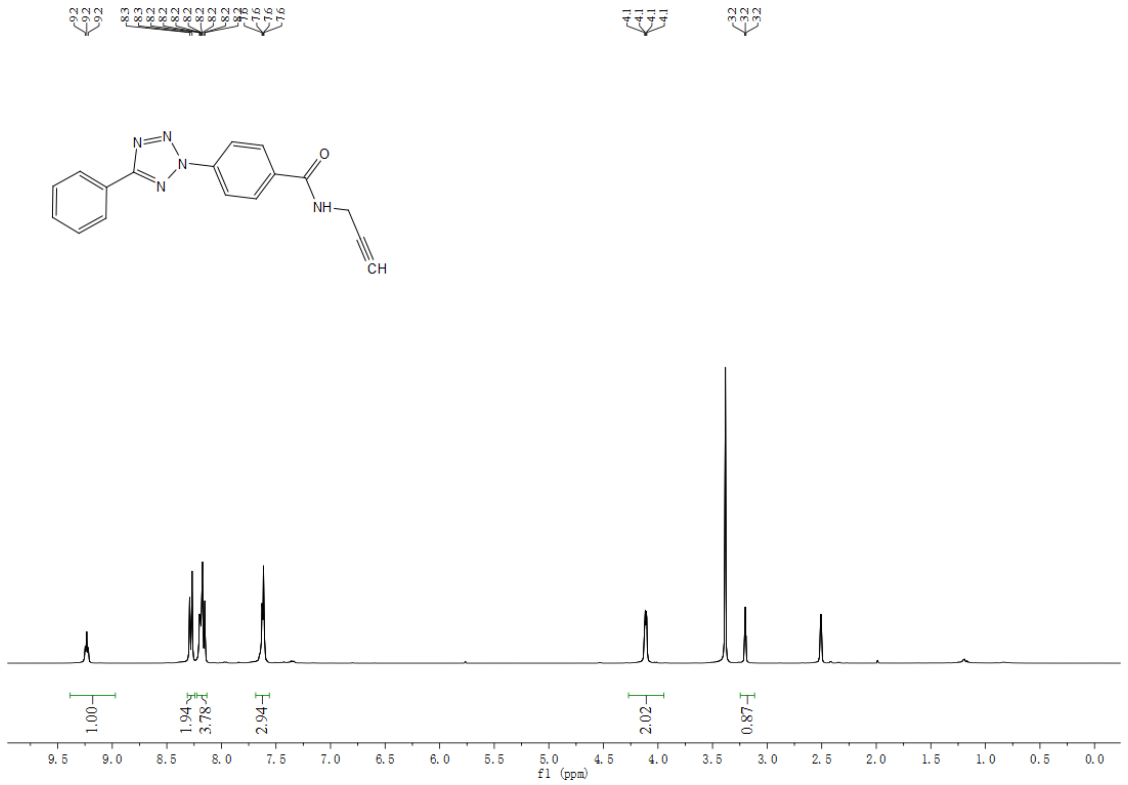
6 ^{1}H NMR



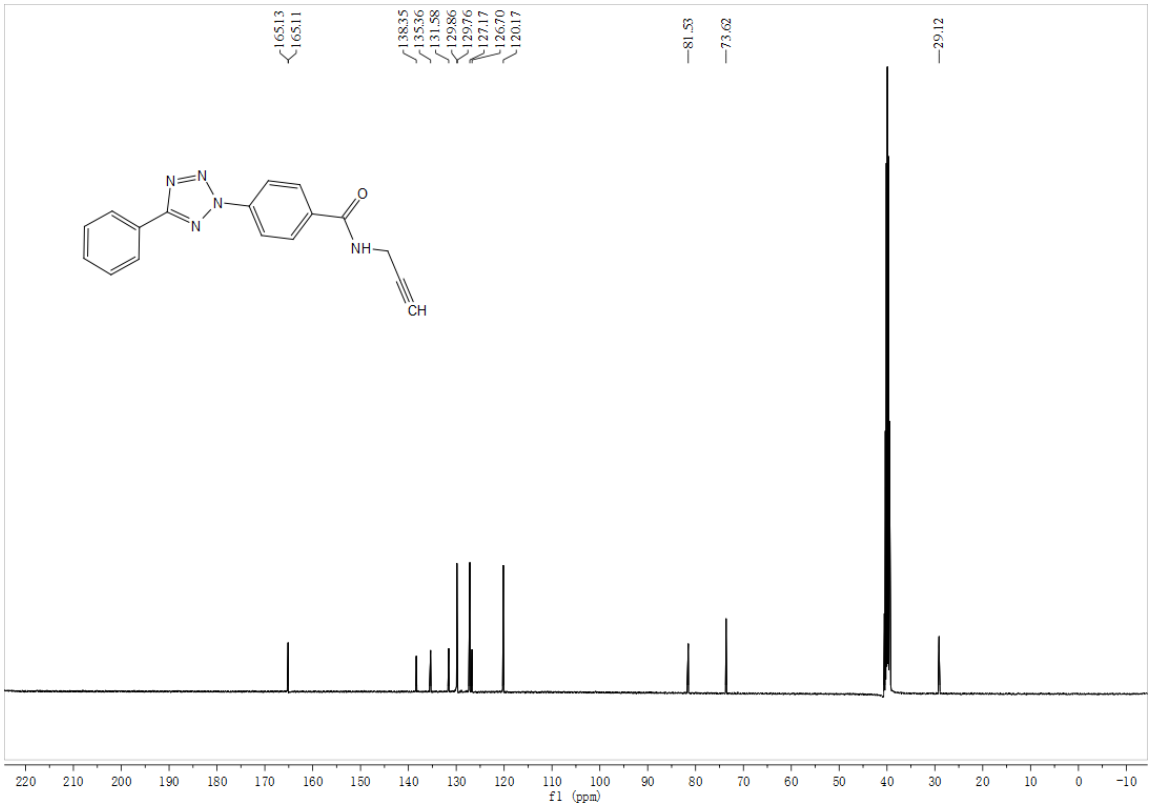
6 ^{13}C NMR



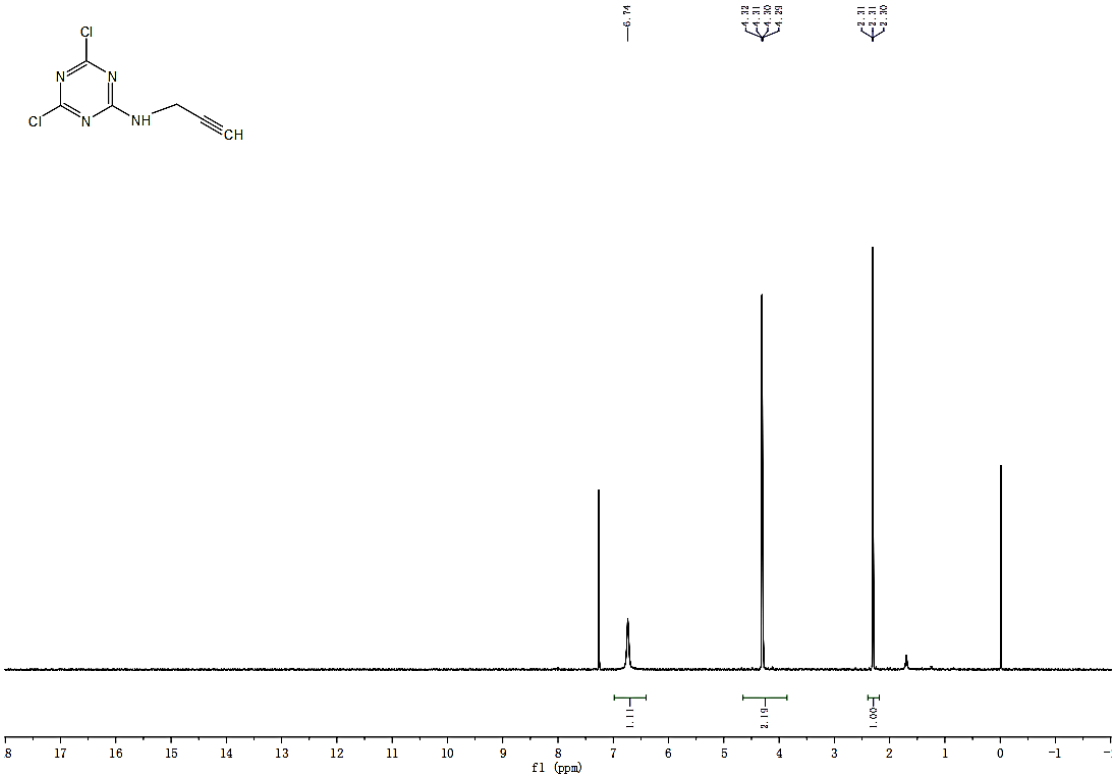
8 ^{1}H NMR



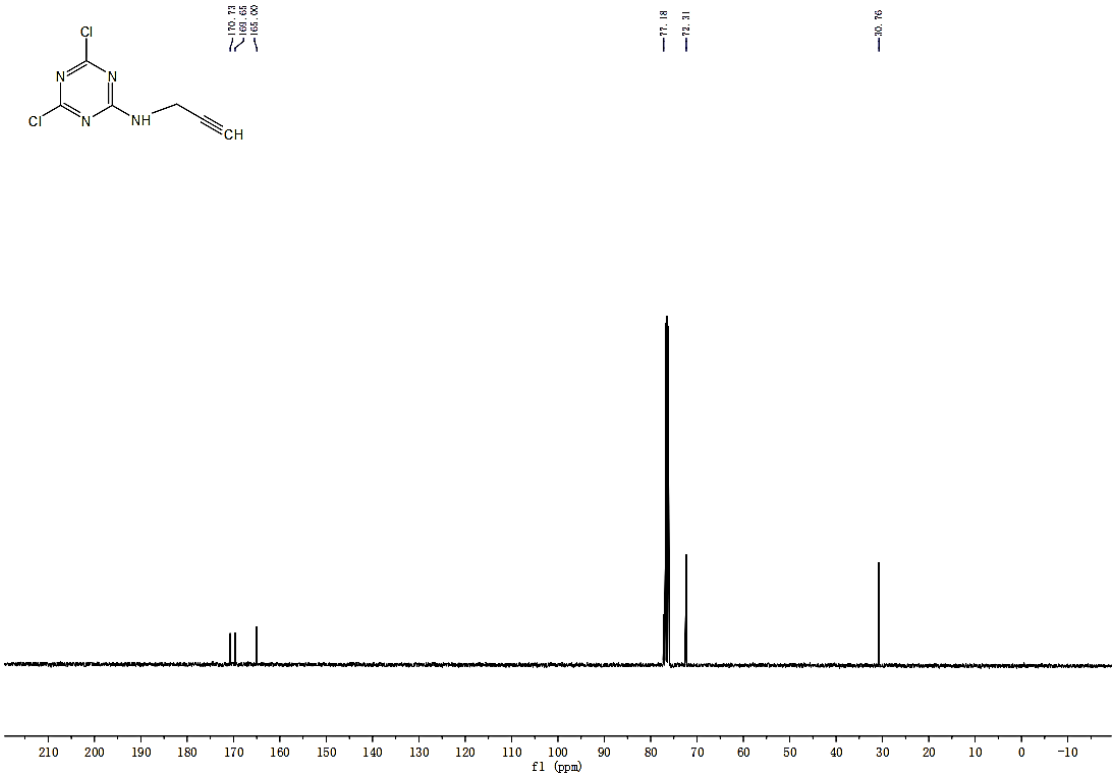
8 ^{13}C NMR



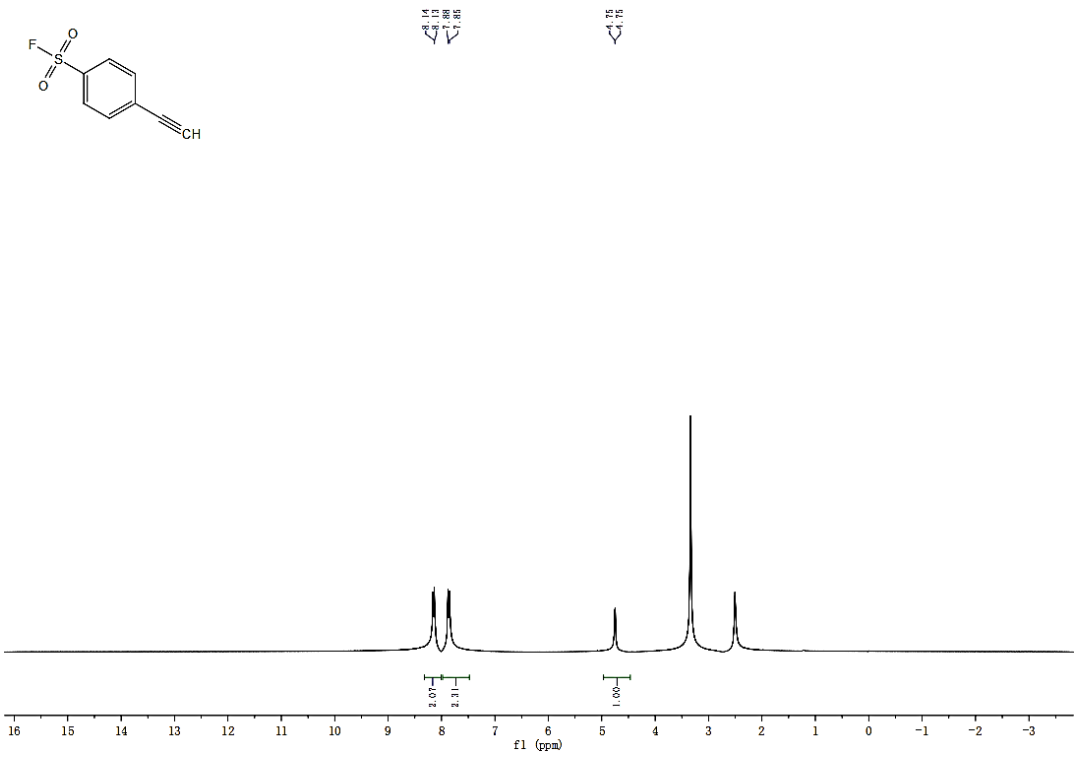
12 ^{1}H NMR



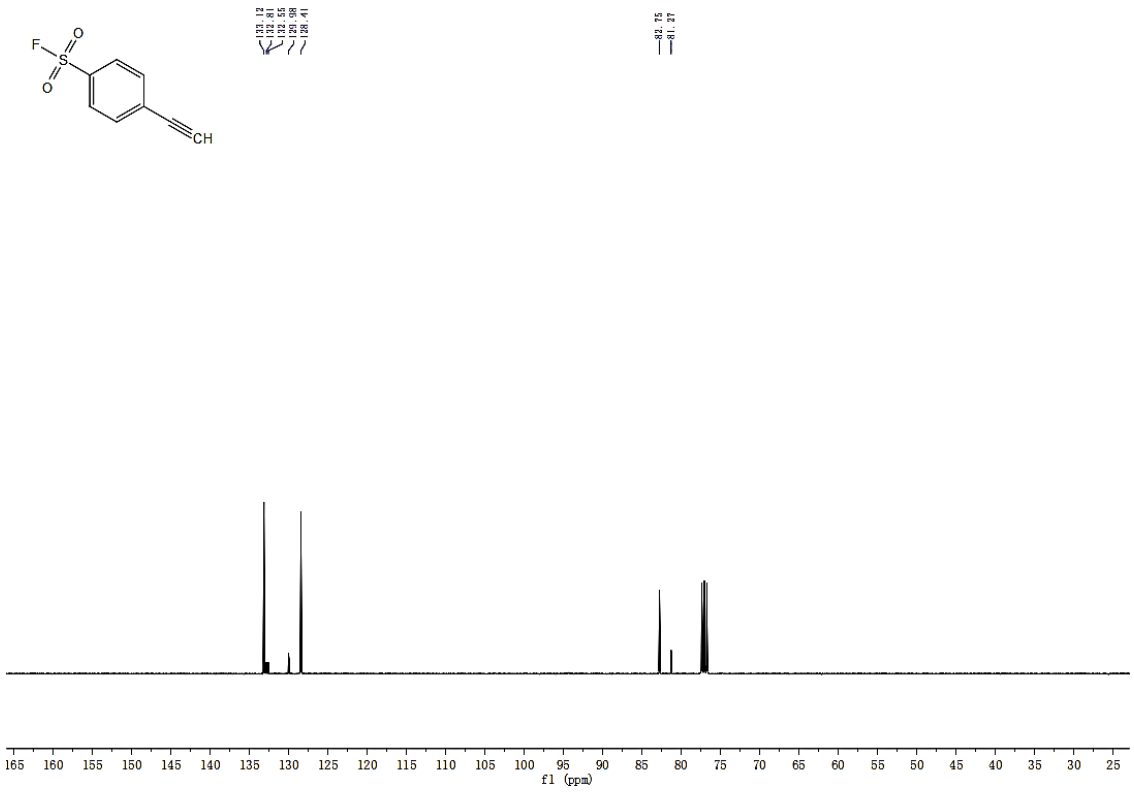
12 ^{13}C NMR



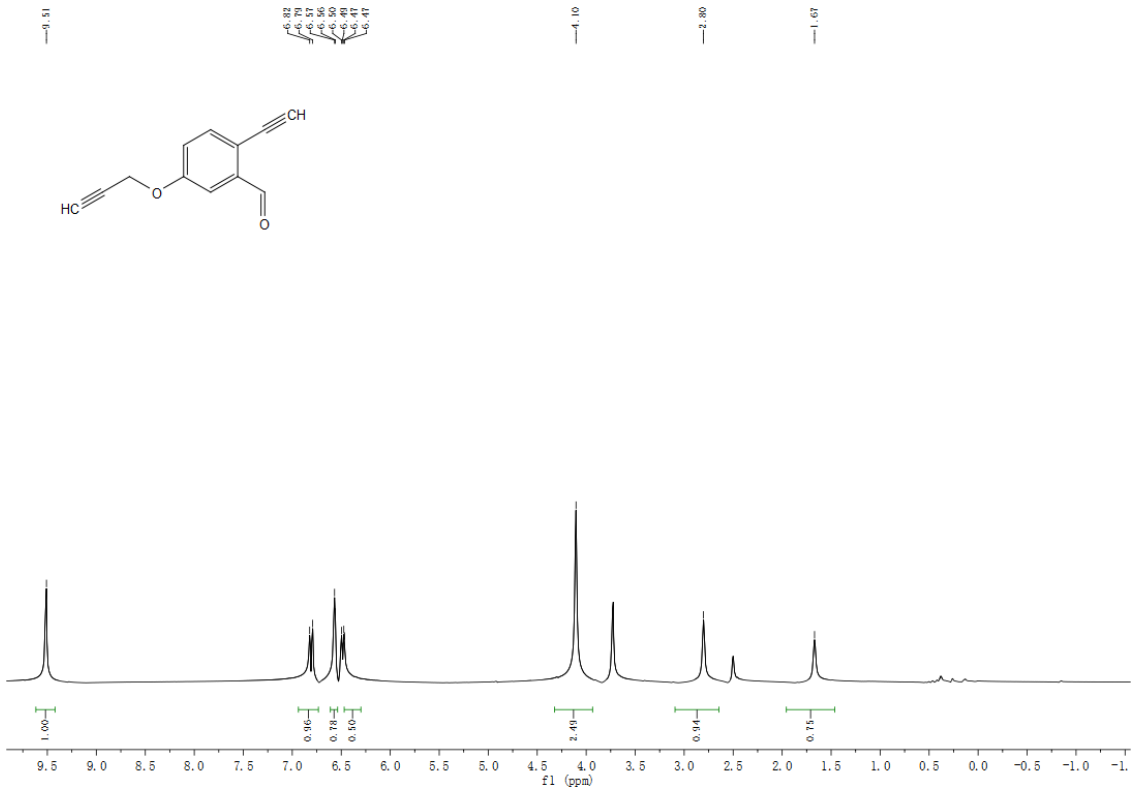
13 ^{1}H NMR



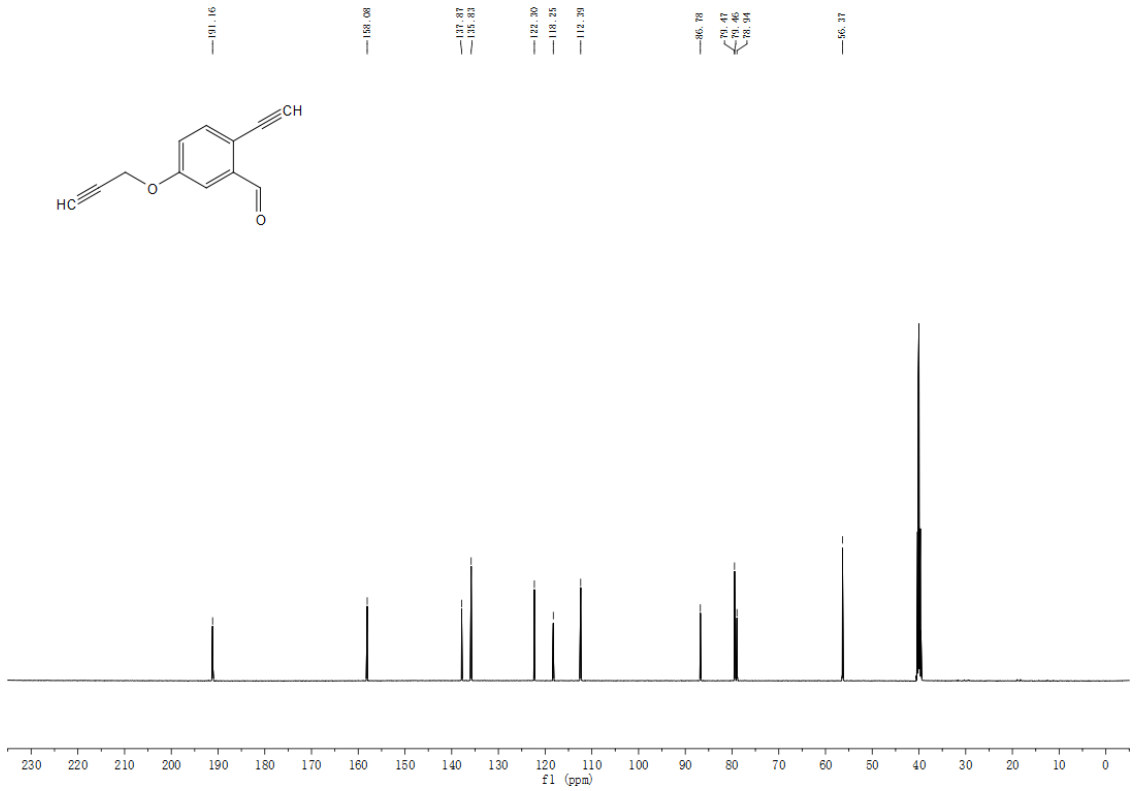
13 ^{13}C NMR



14 ^{1}H NMR

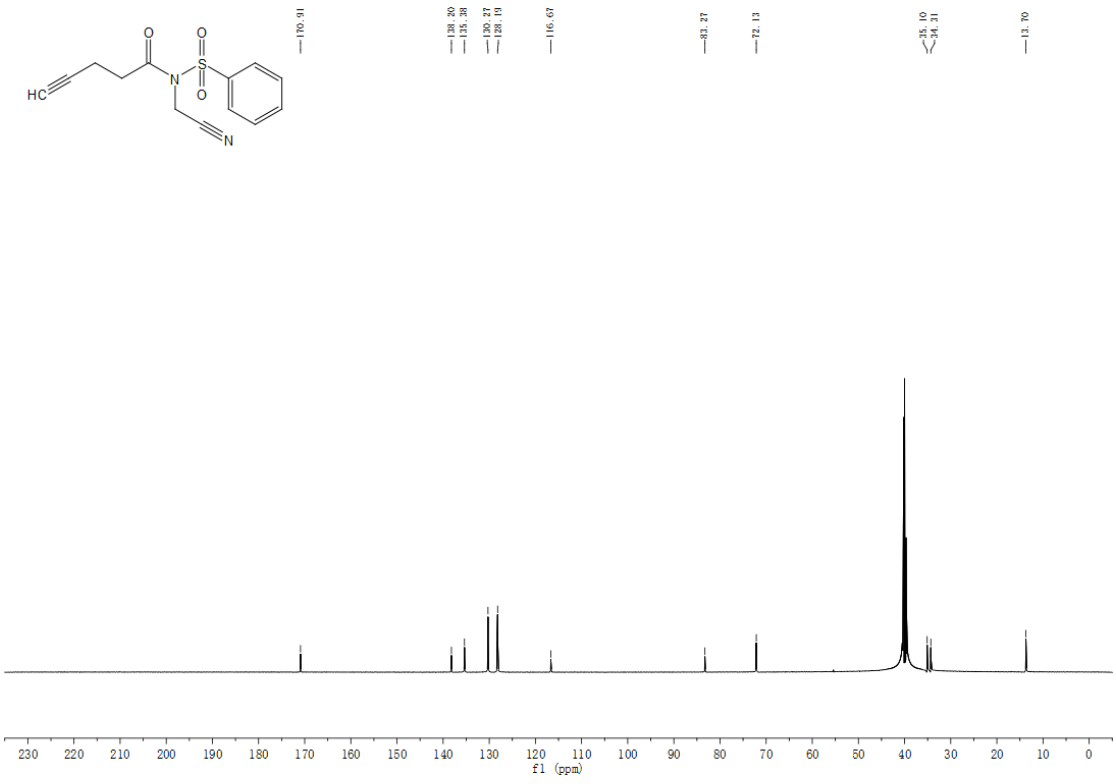


14 ^{13}C NMR

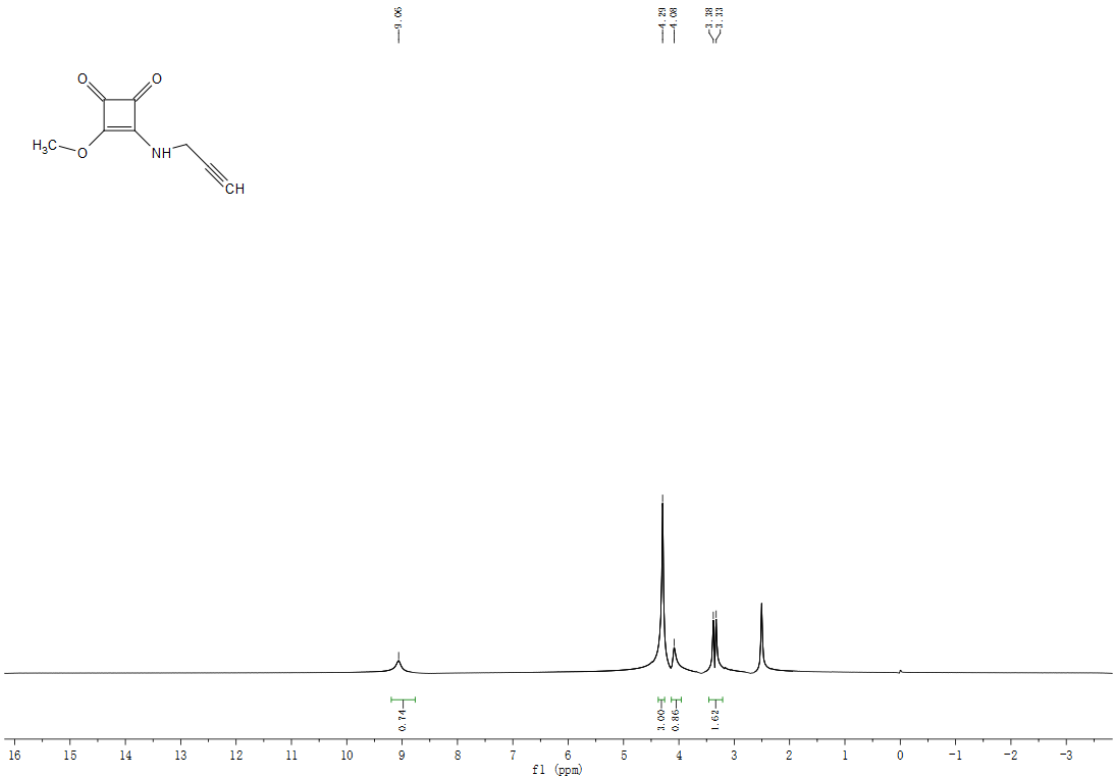


15 ^{1}H NMR

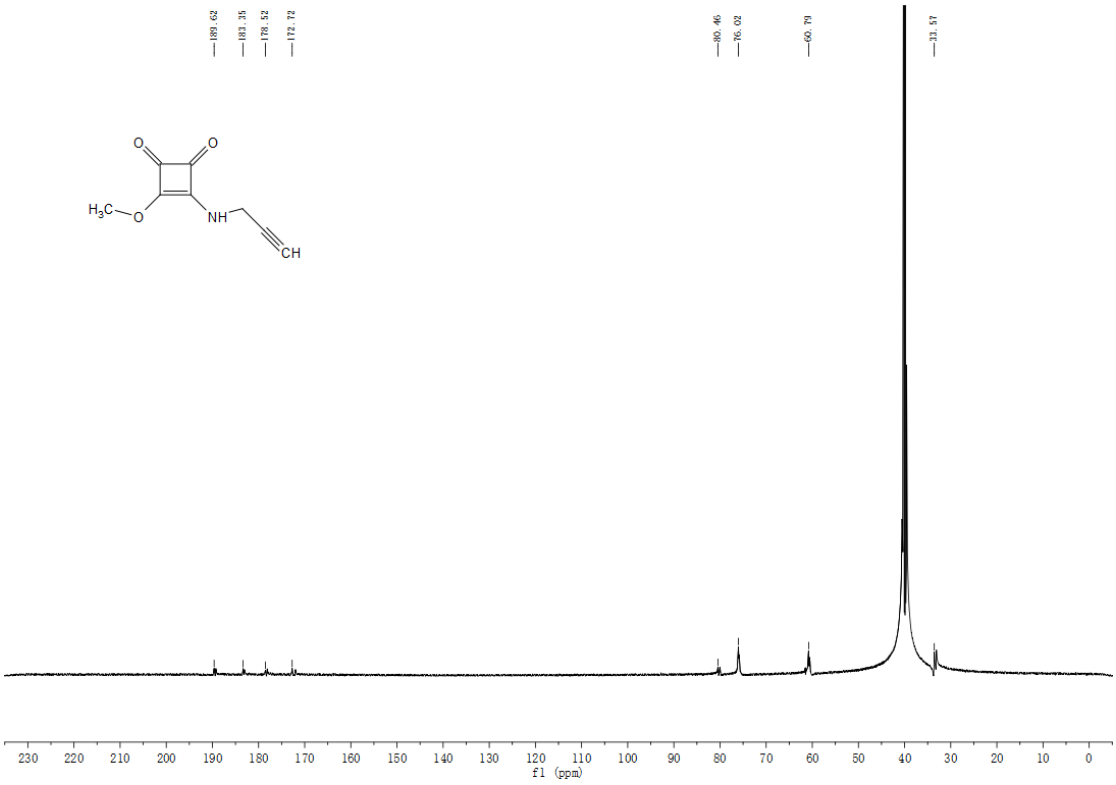
15 ^{13}C NMR



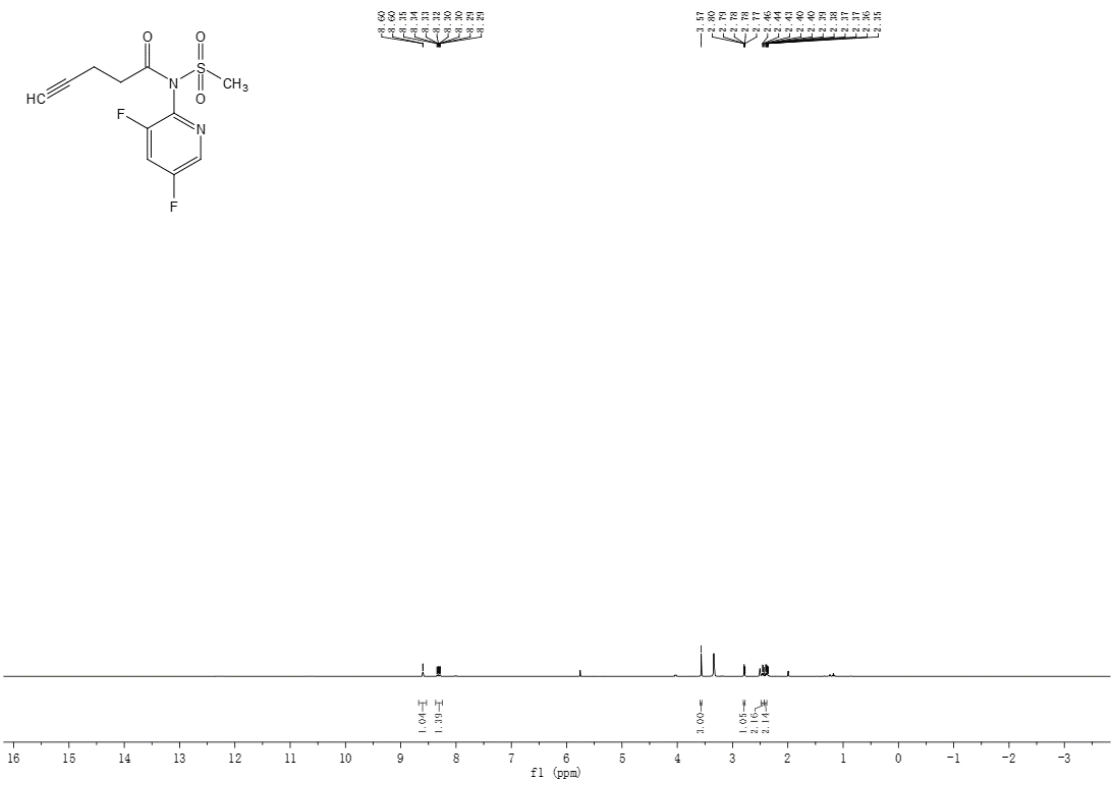
16 ^{1}H NMR



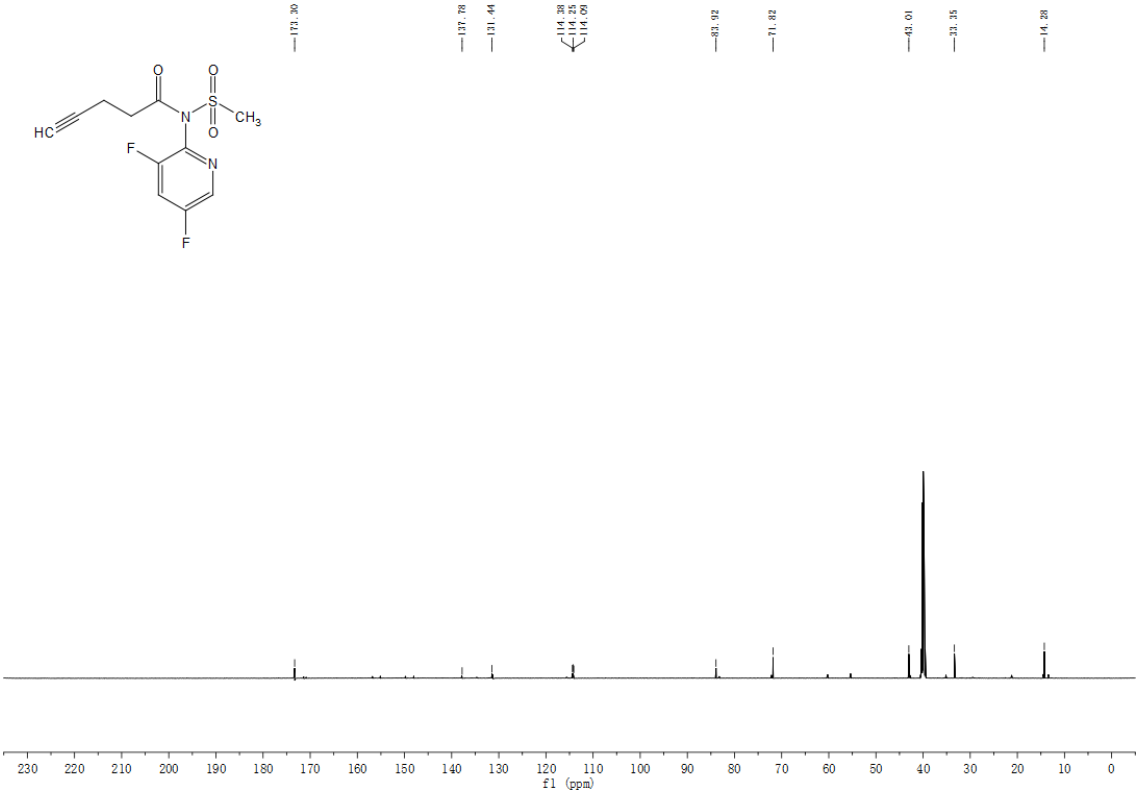
16 ^{13}C NMR



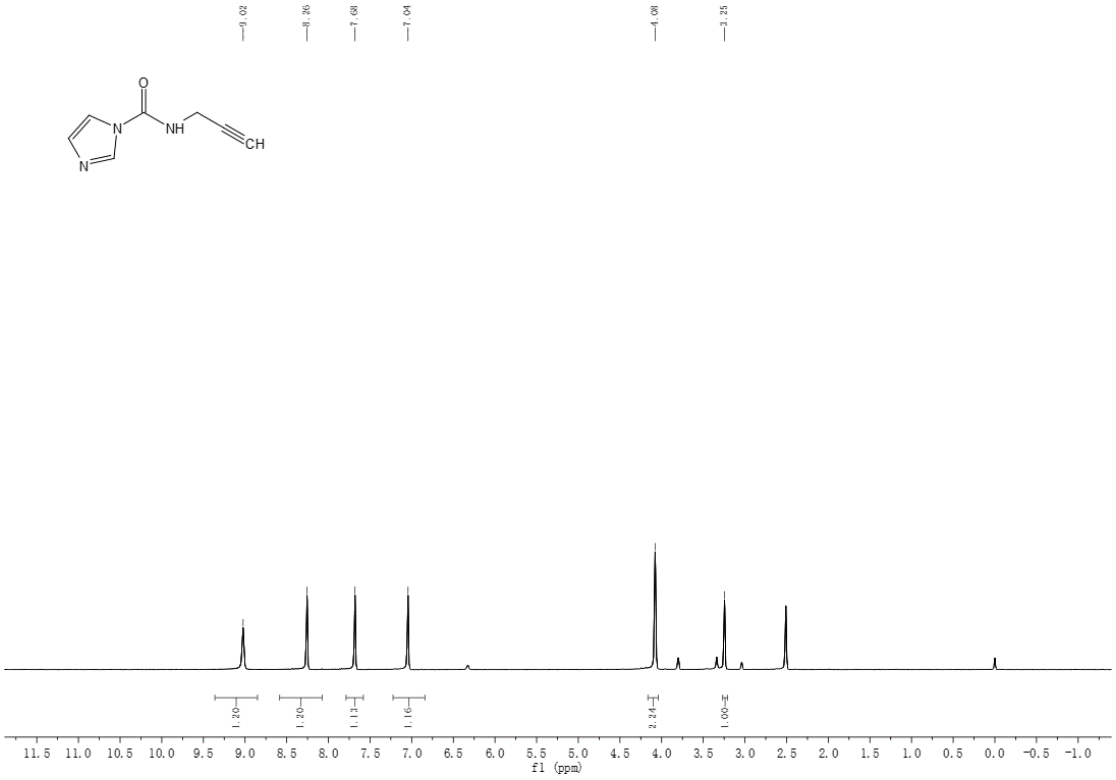
17 ^{1}H NMR



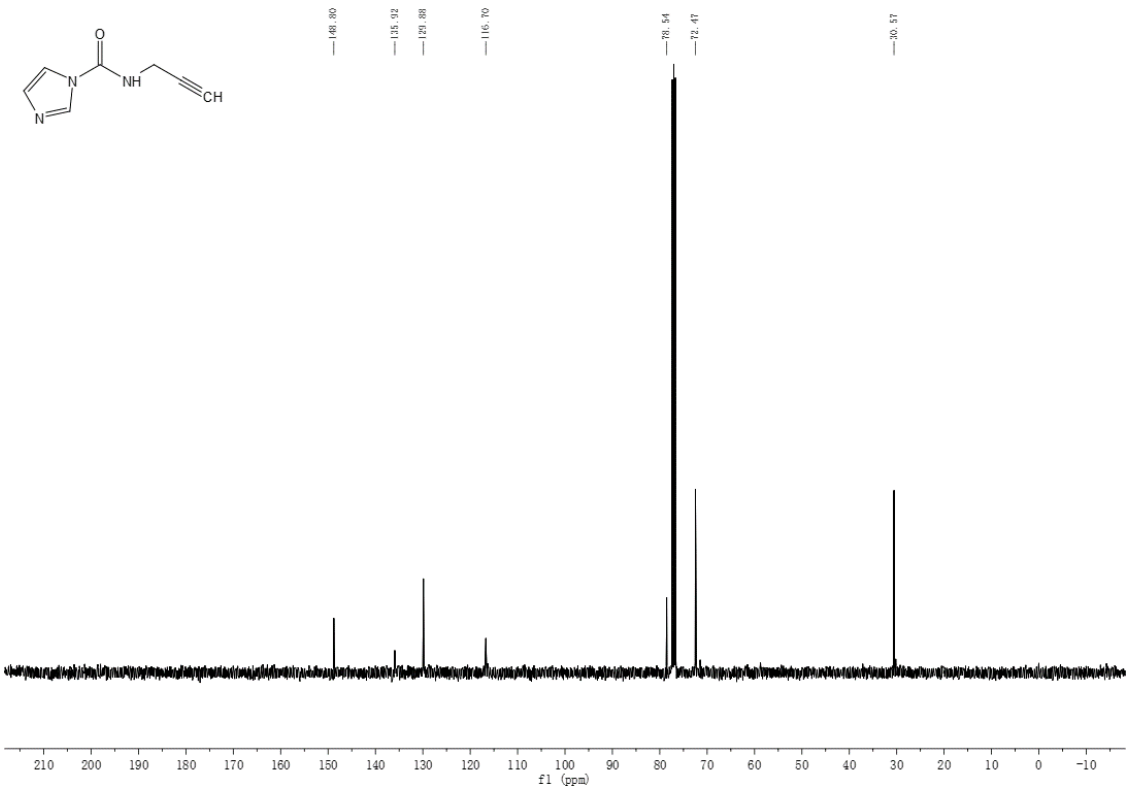
17 ^{13}C NMR



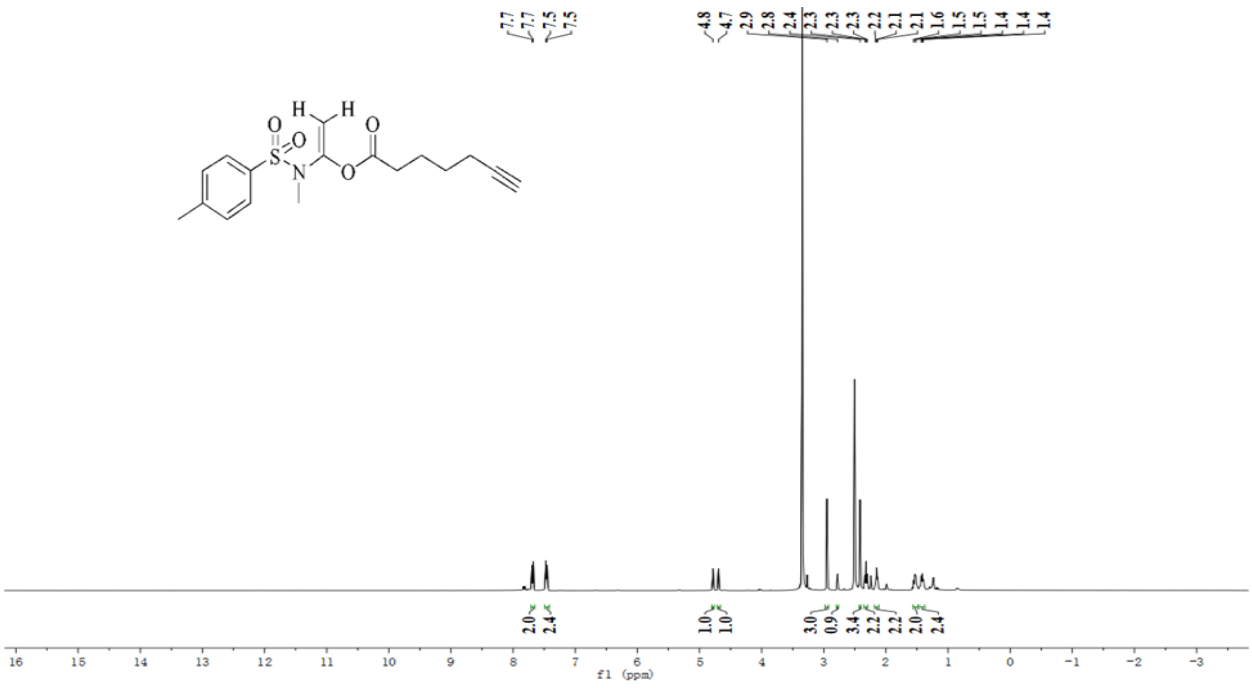
18 ^{1}H NMR



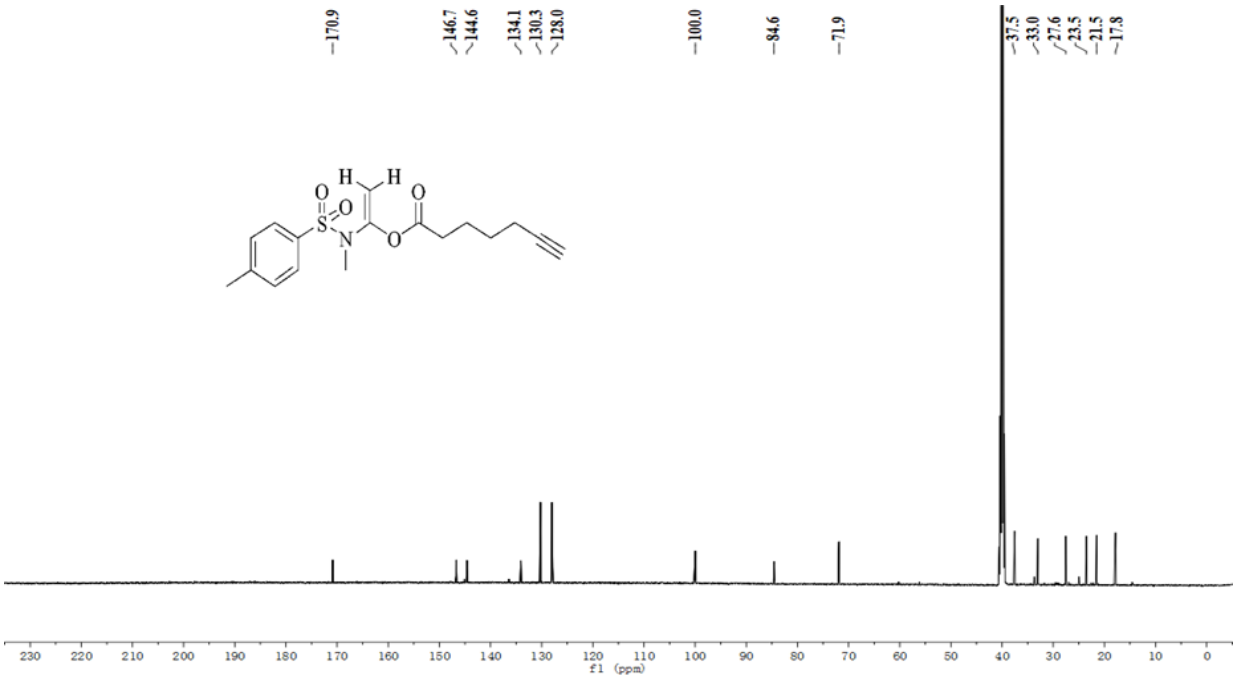
18 ^{13}C NMR



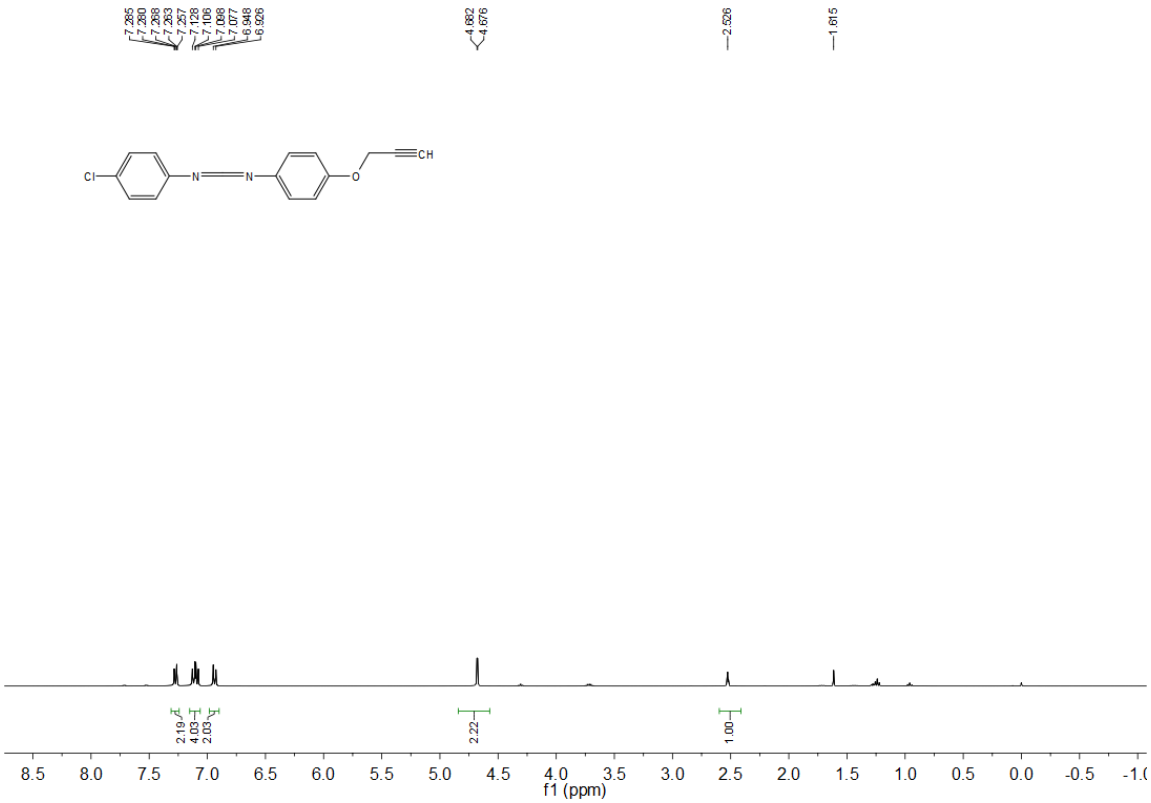
19 ^{1}H NMR



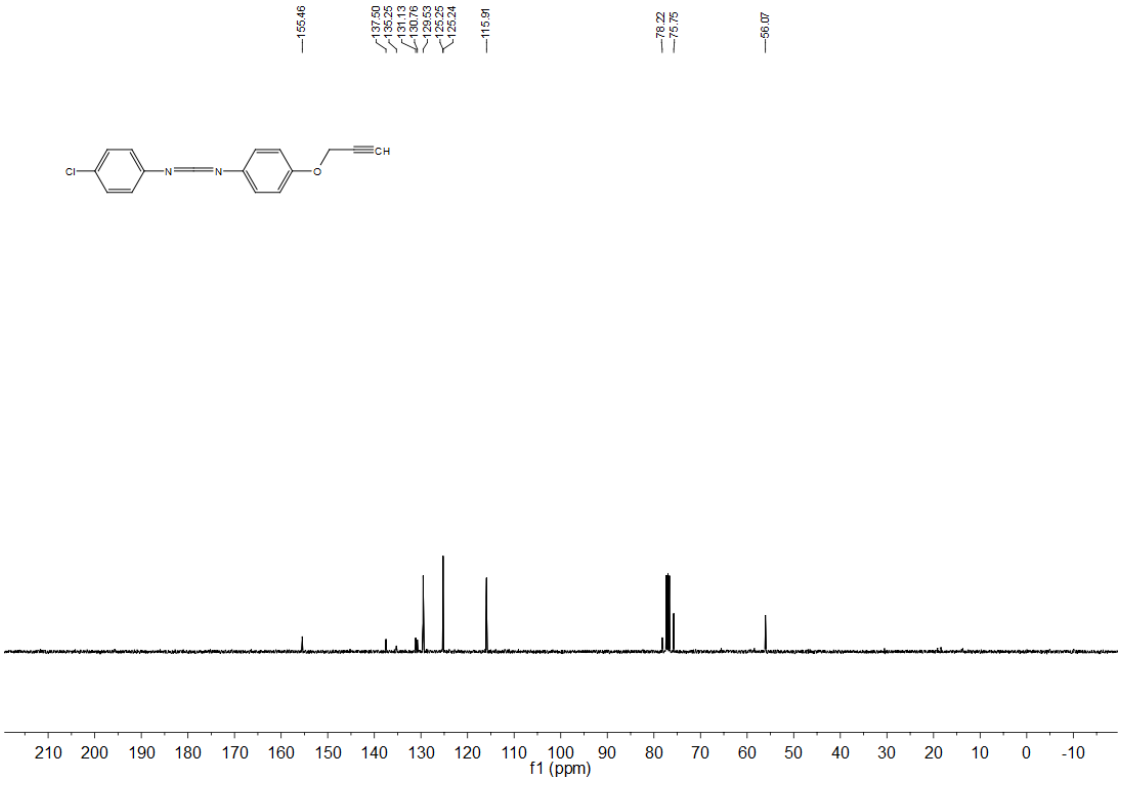
19 ^{13}C NMR



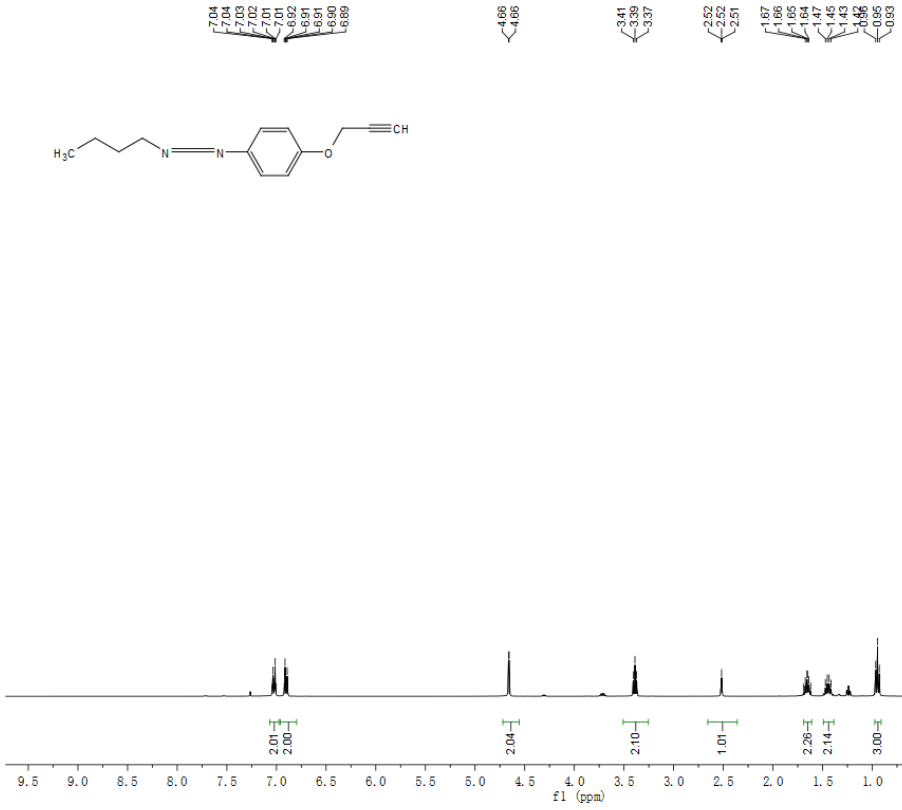
20 ^{1}H NMR



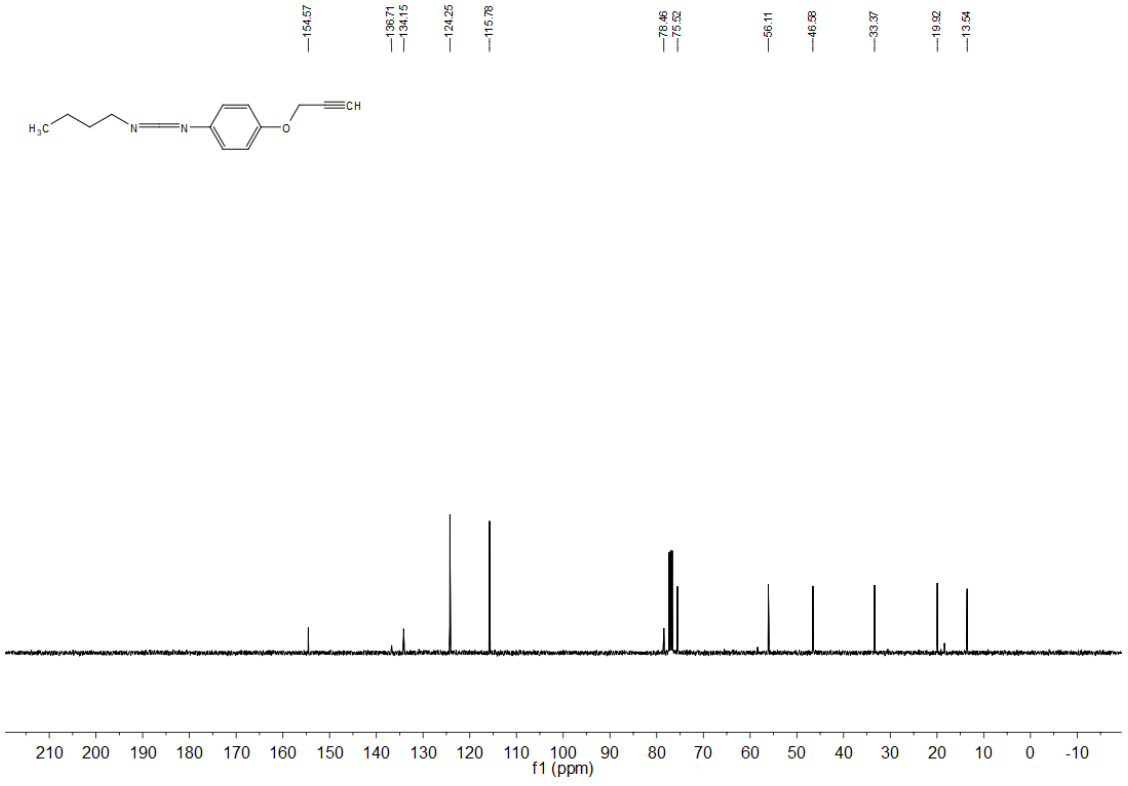
20 ^{13}C NMR



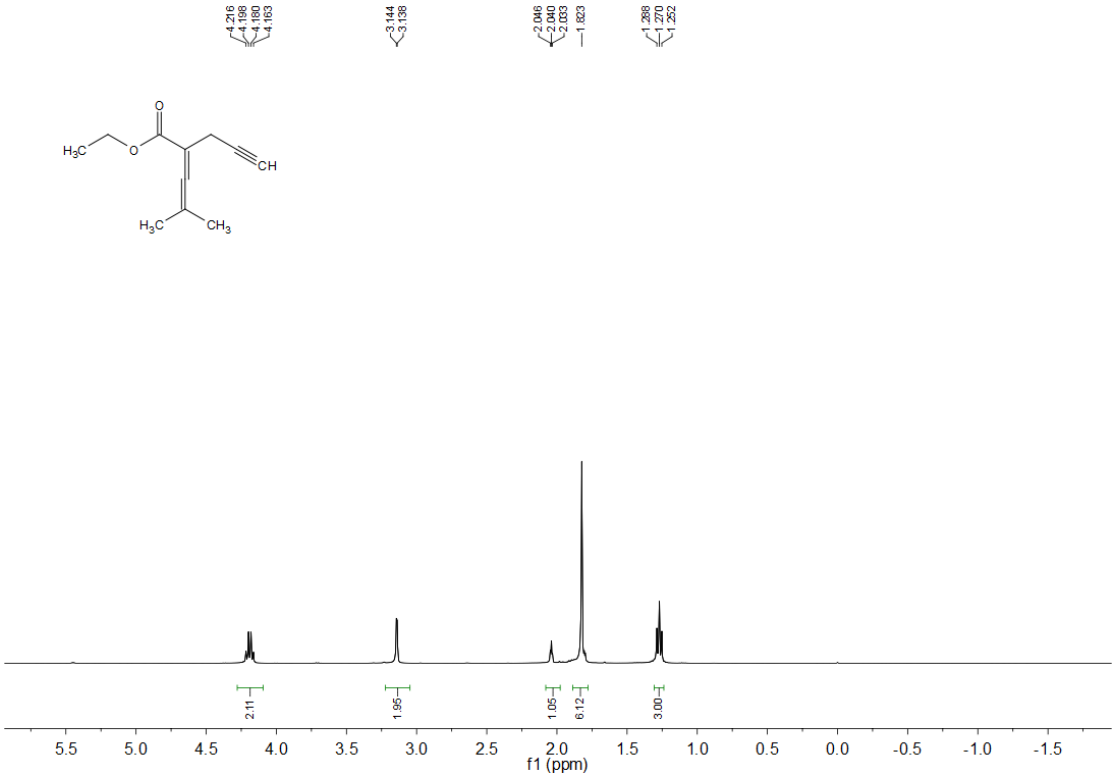
21 ^{1}H NMR



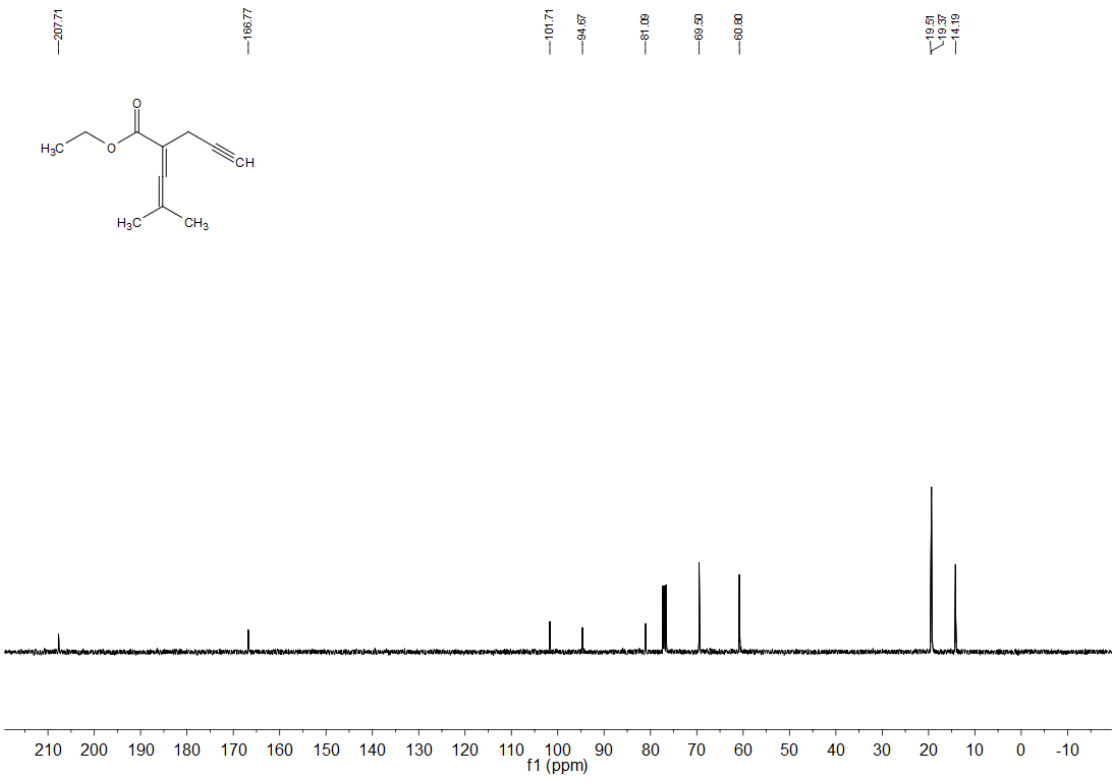
21 ^{13}C NMR



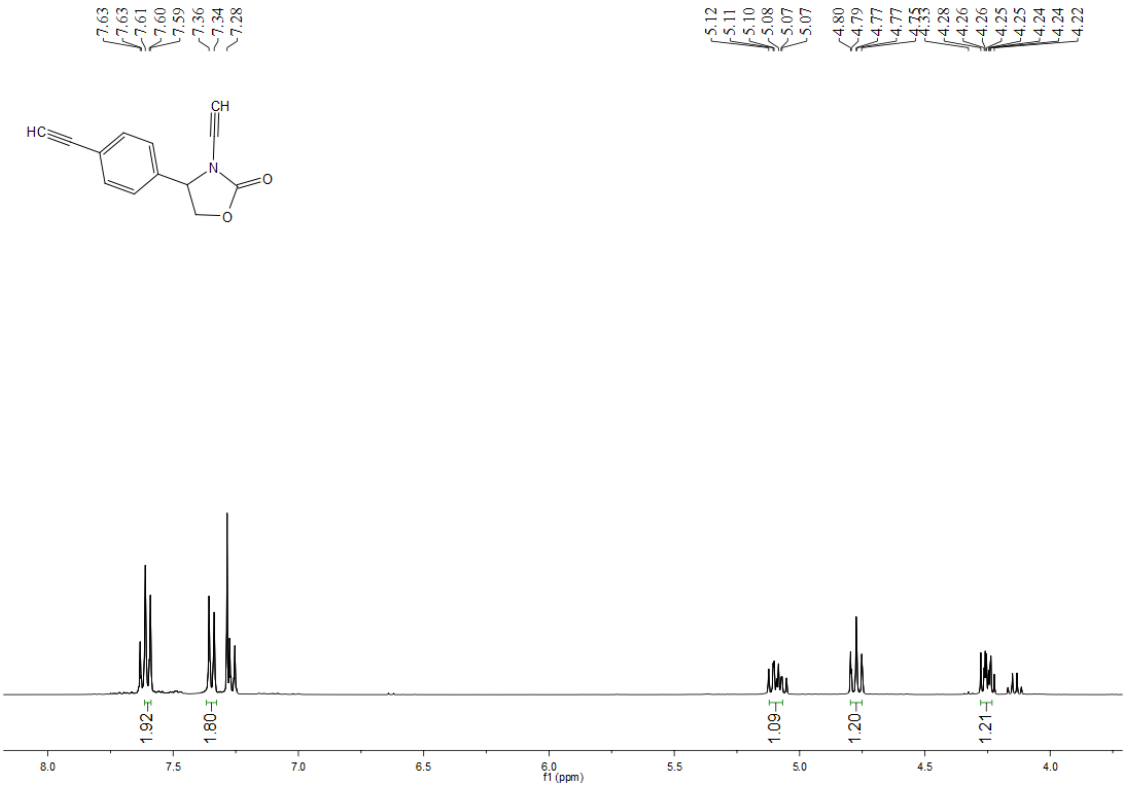
22 ^{1}H NMR



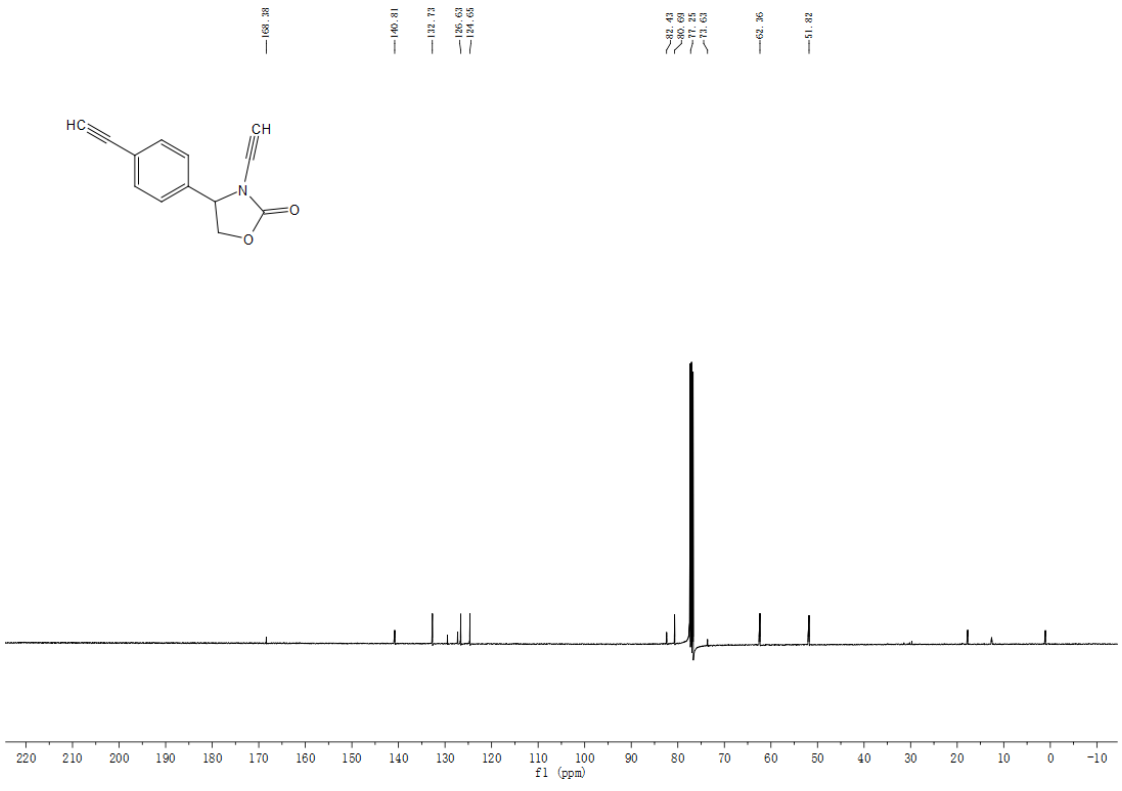
22 ^{13}C NMR



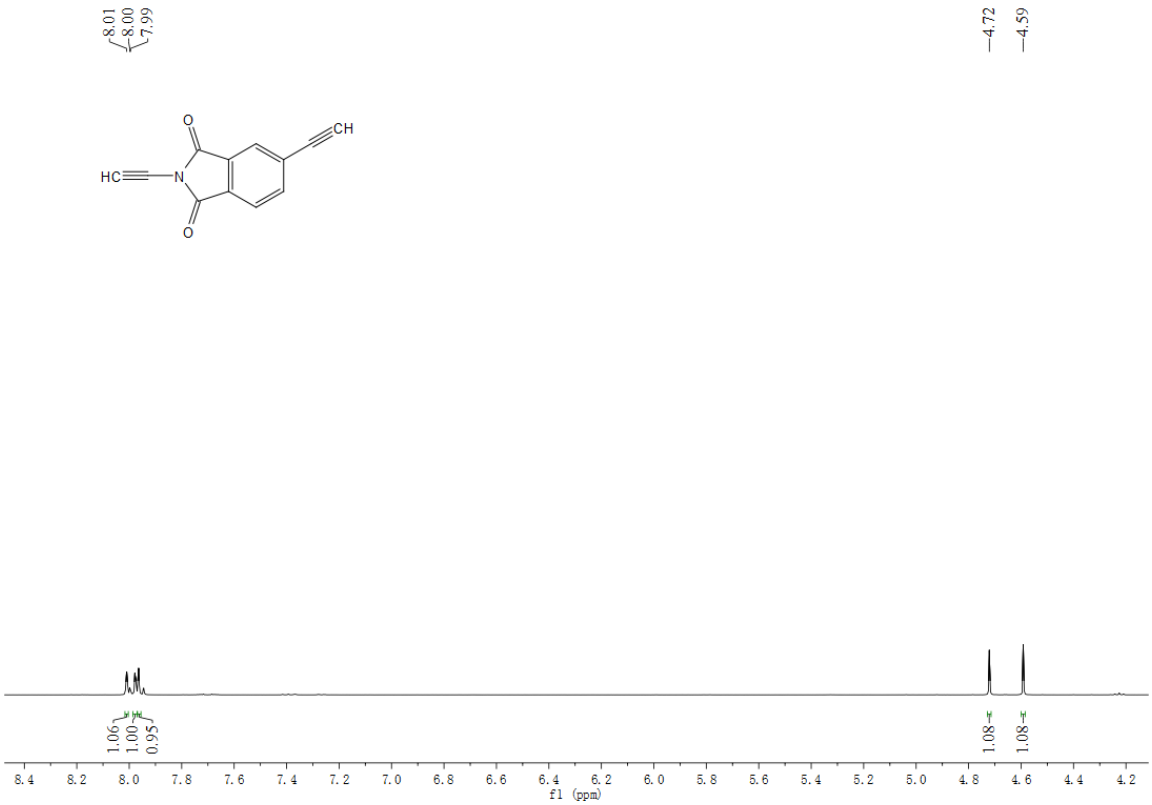
23 ^{1}H NMR



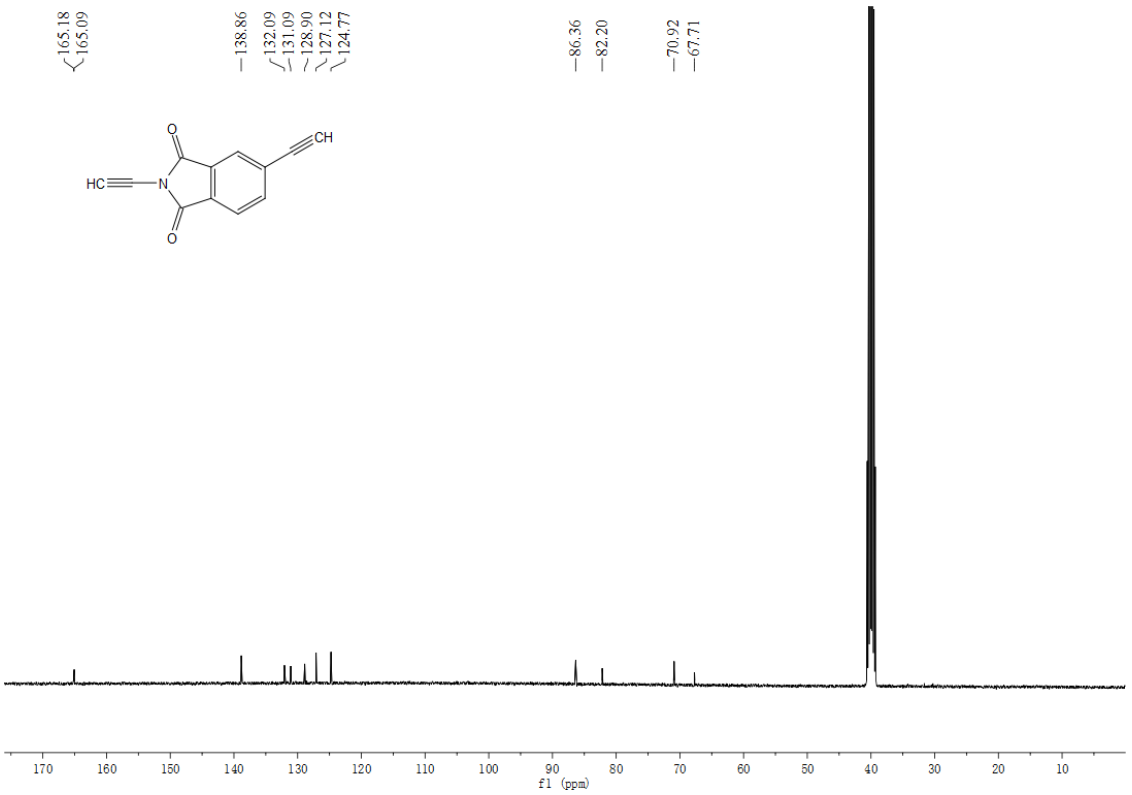
23 ^{13}C NMR



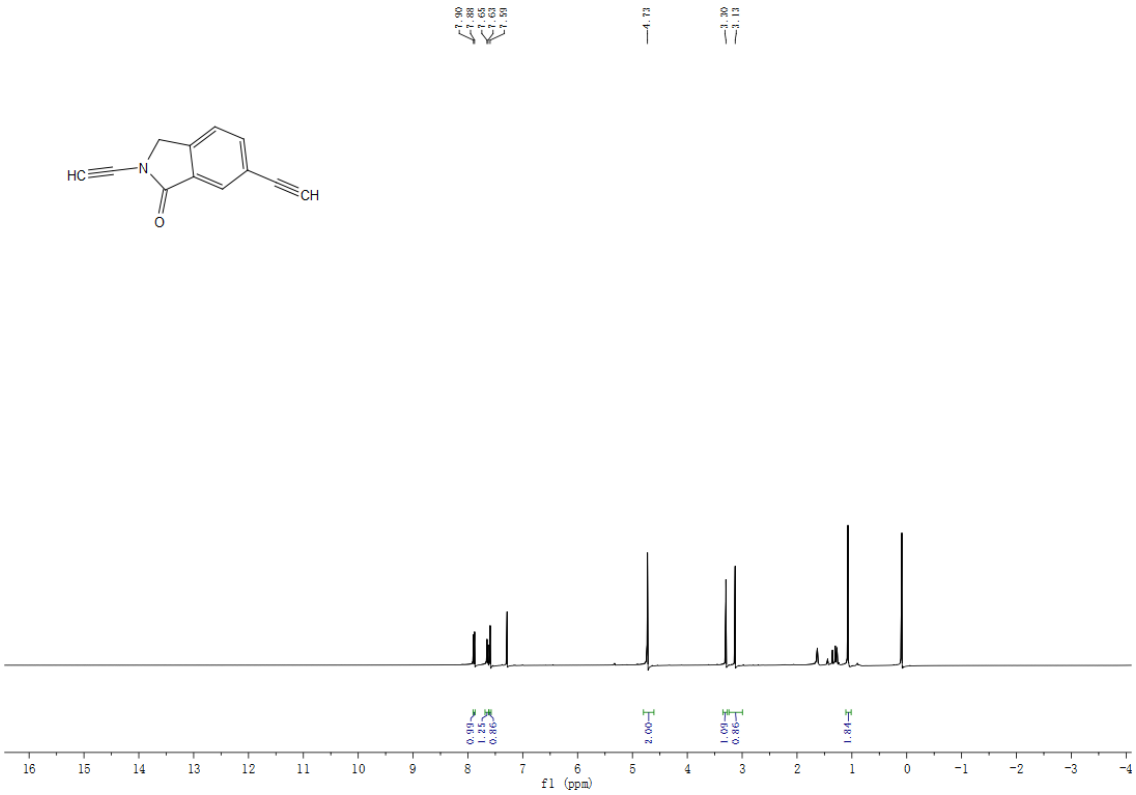
24 ^{1}H NMR



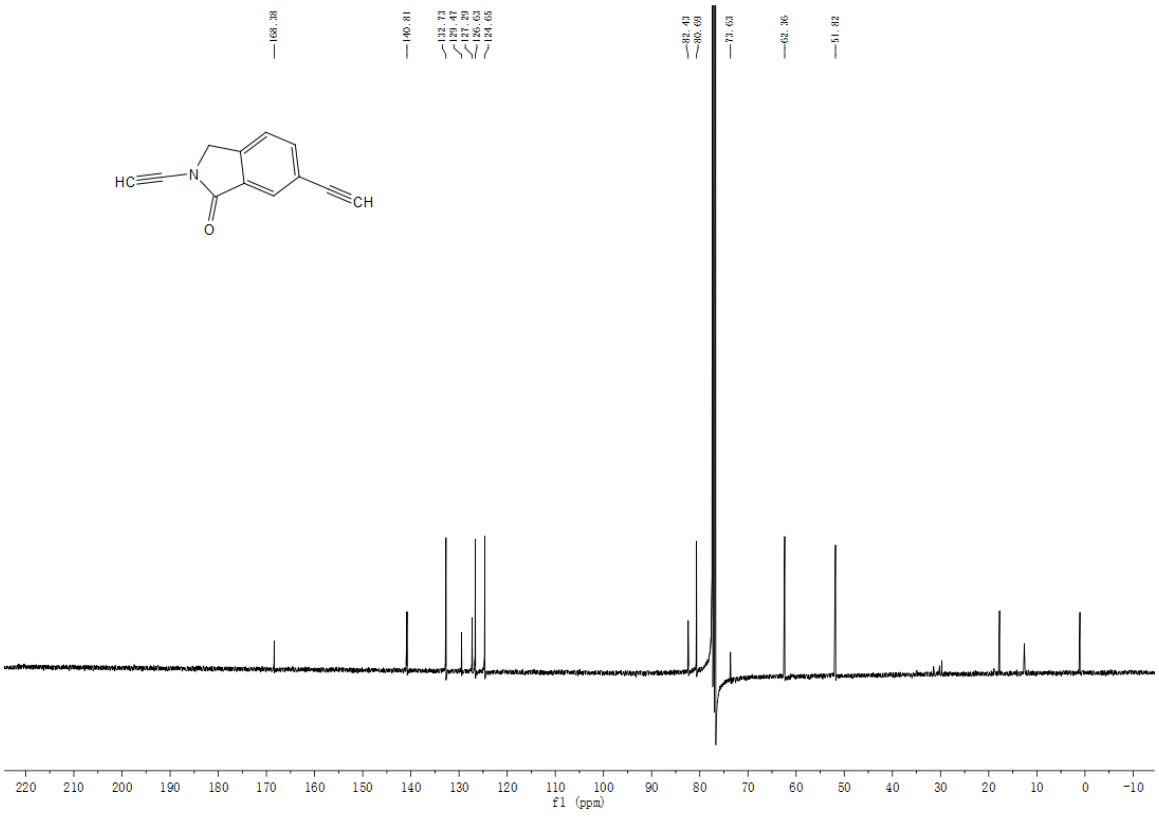
24 ^{13}C NMR



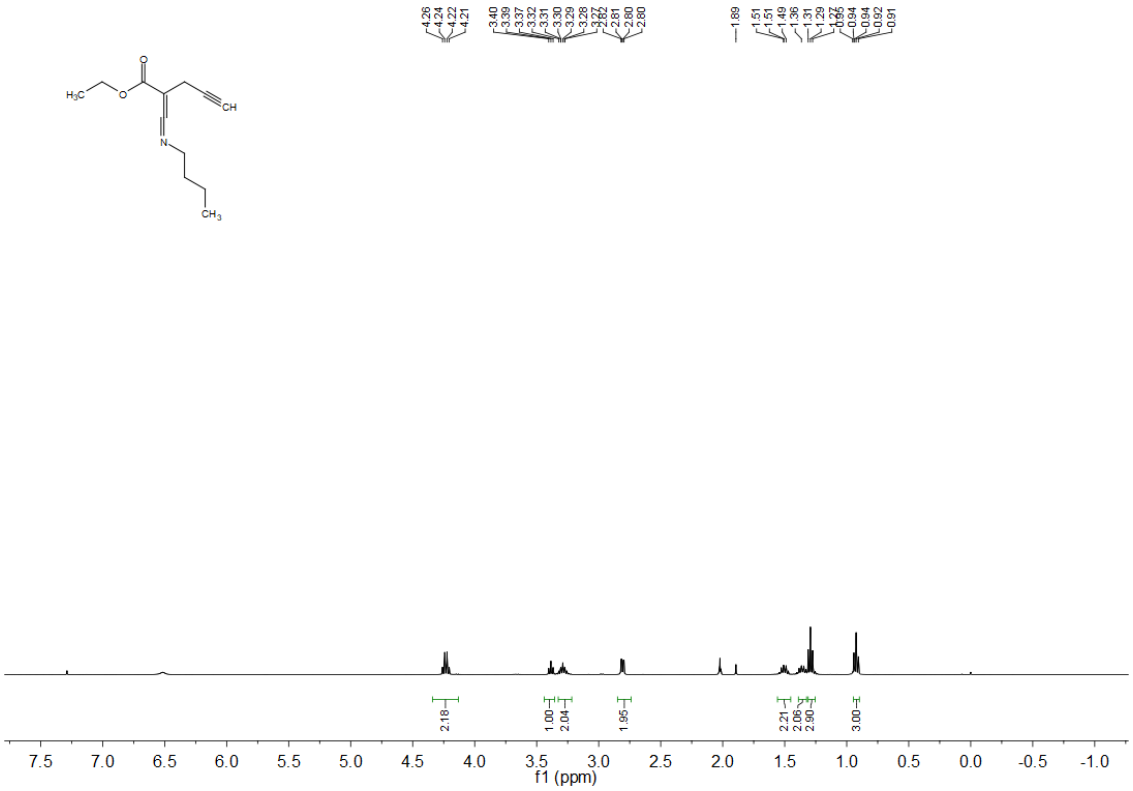
25 ^{1}H NMR



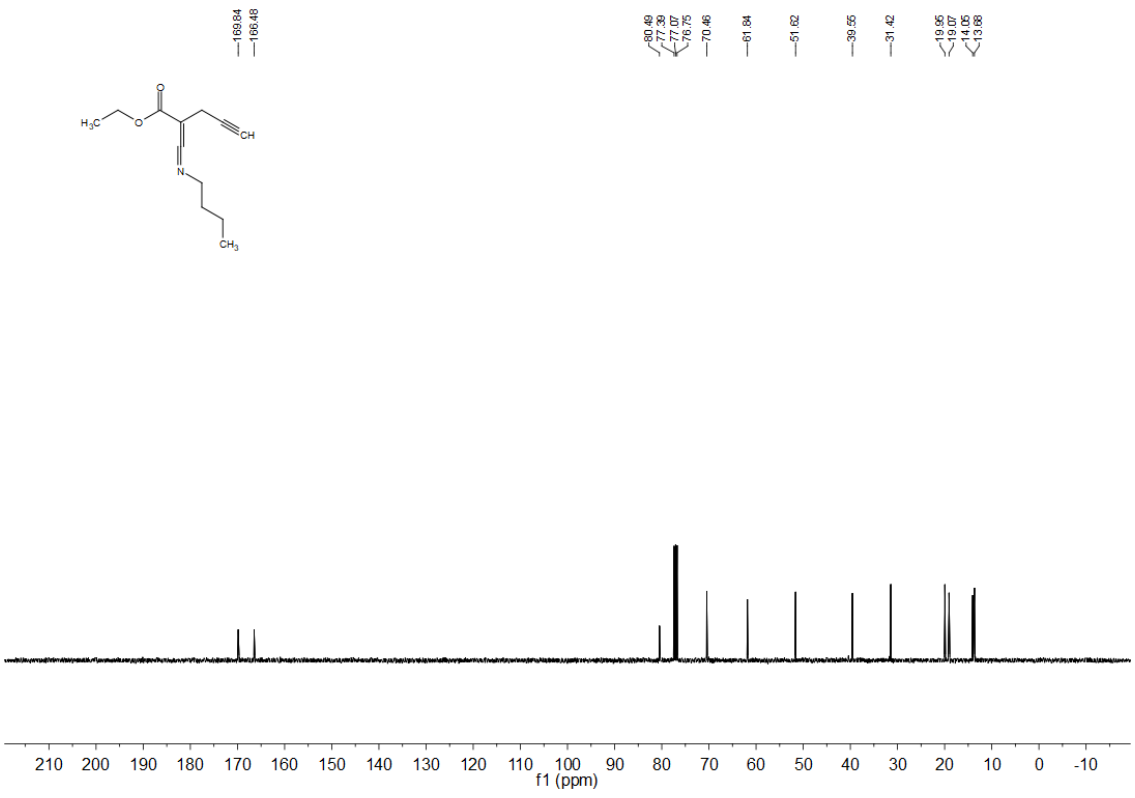
25^{13}C NMR



26 ^{1}H NMR

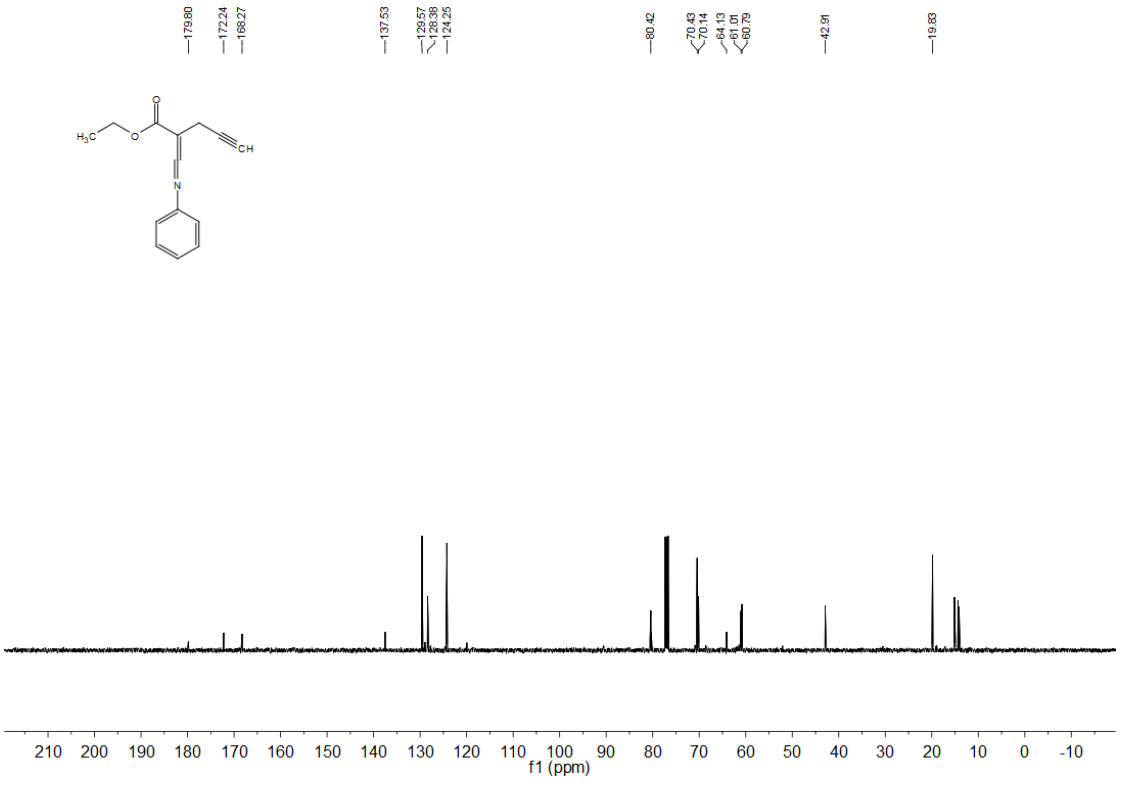


26 ^{13}C NMR

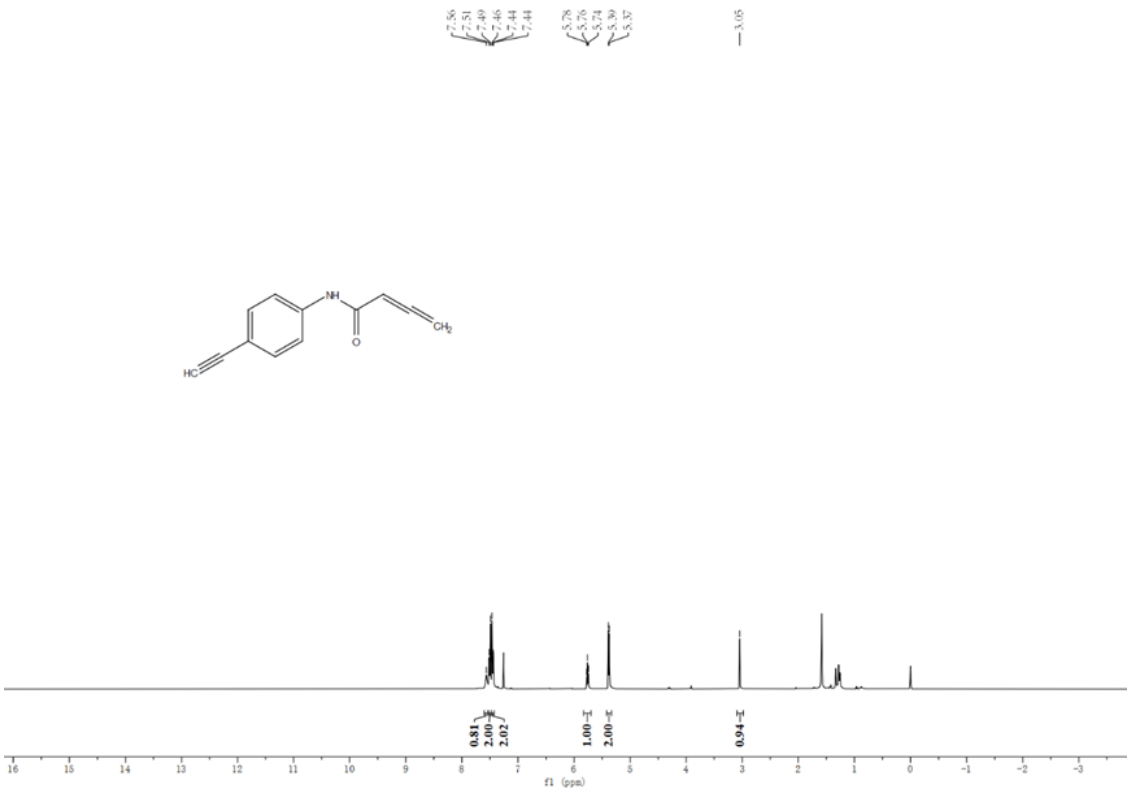


27 ^{1}H NMR

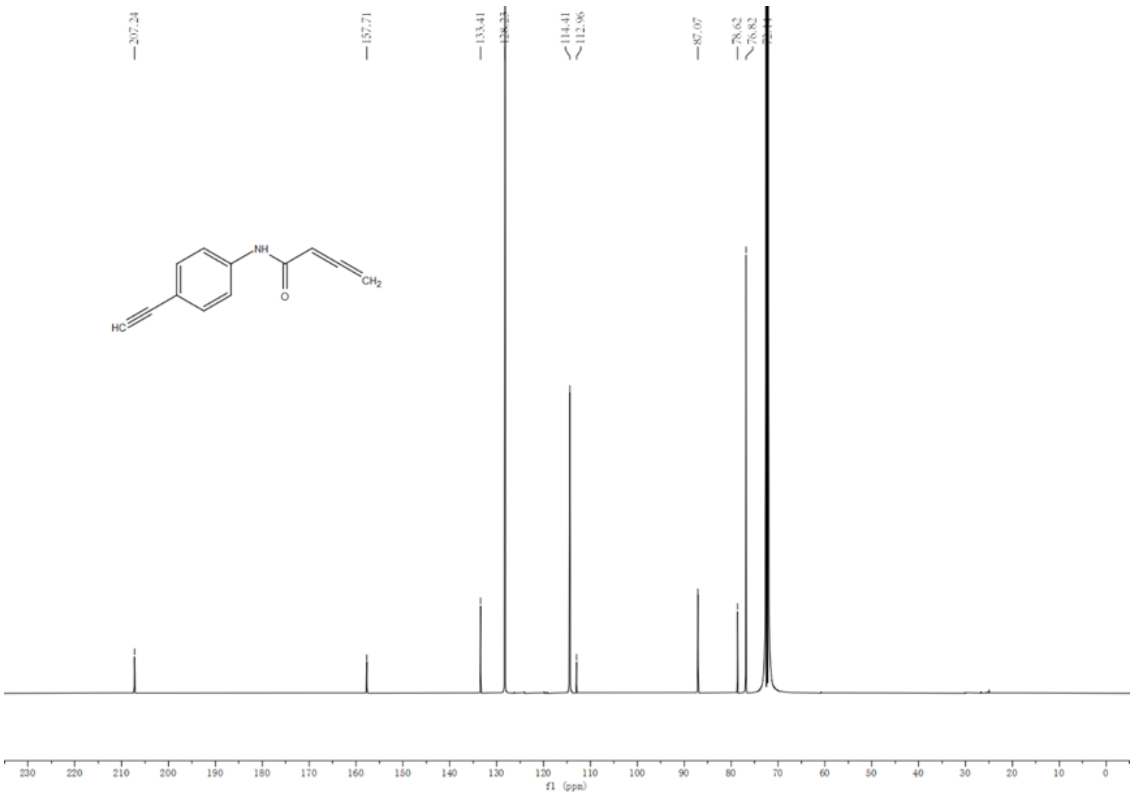
27 ^{13}C NMR



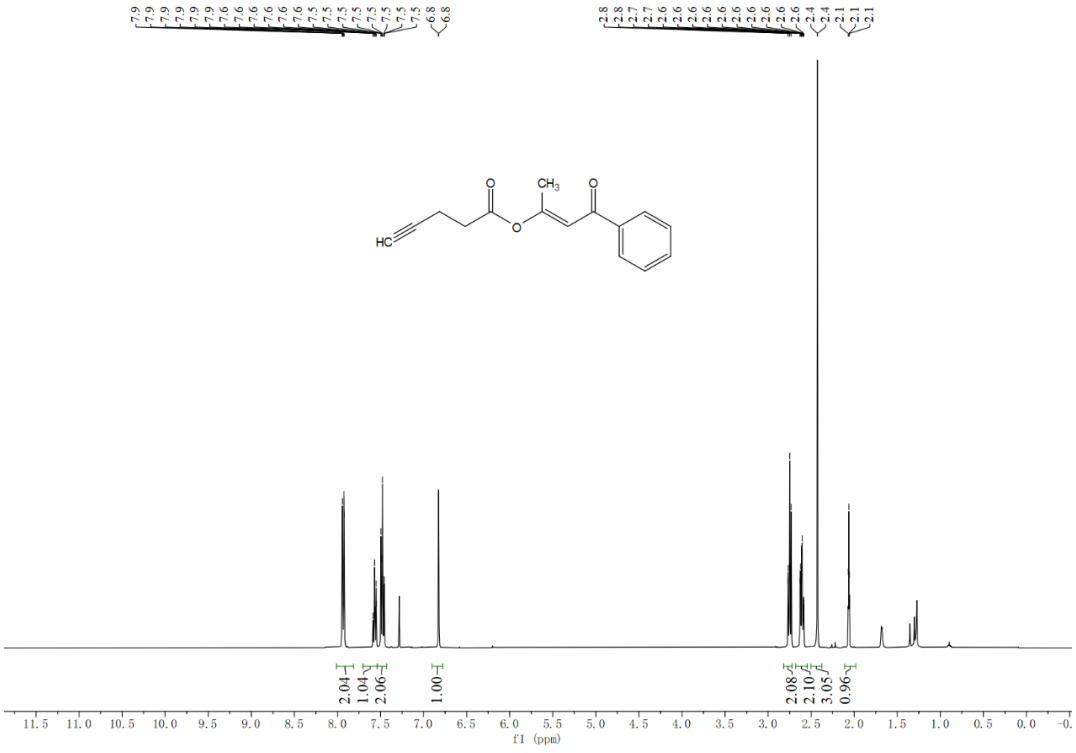
28 ^{1}H NMR



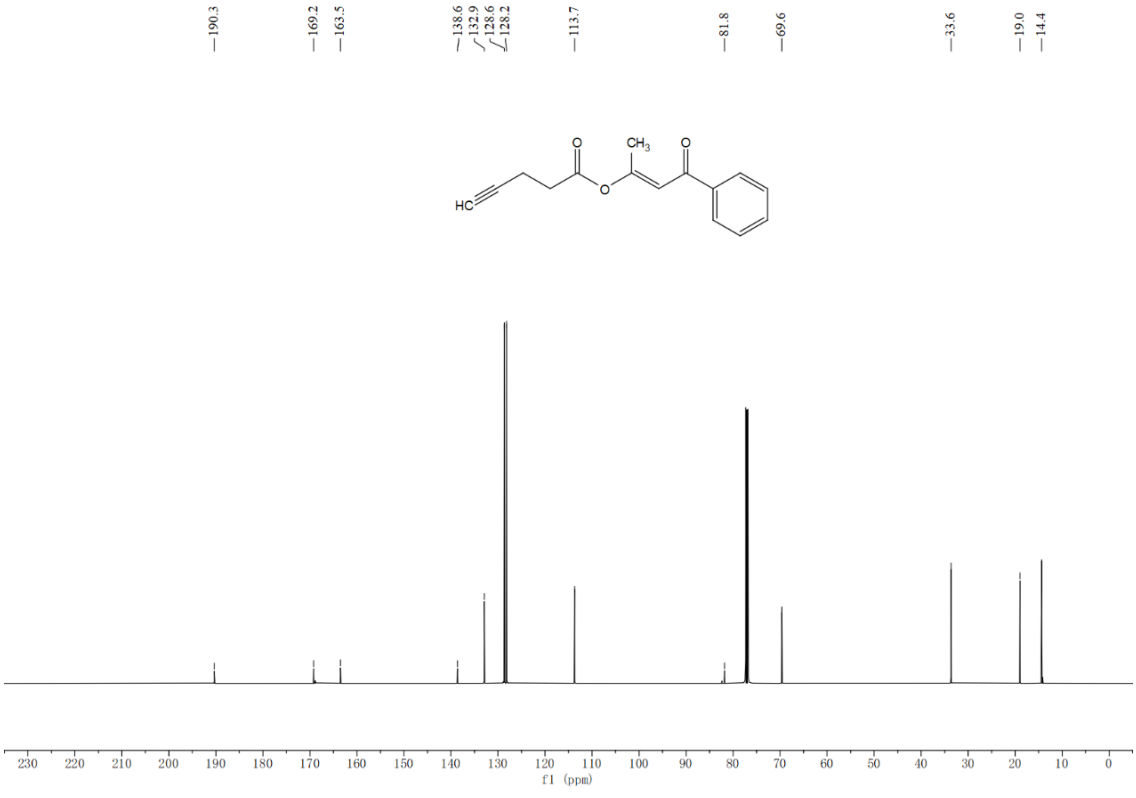
28 ^{13}C NMR



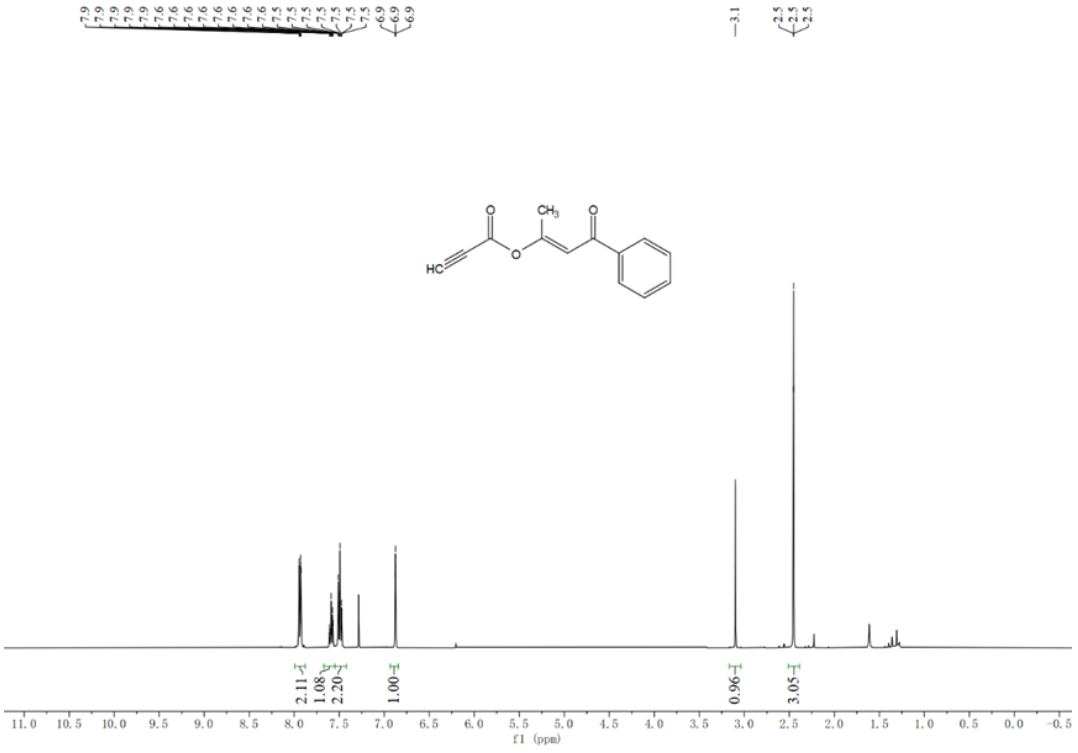
29 ^{1}H NMR



29 ^{13}C NMR

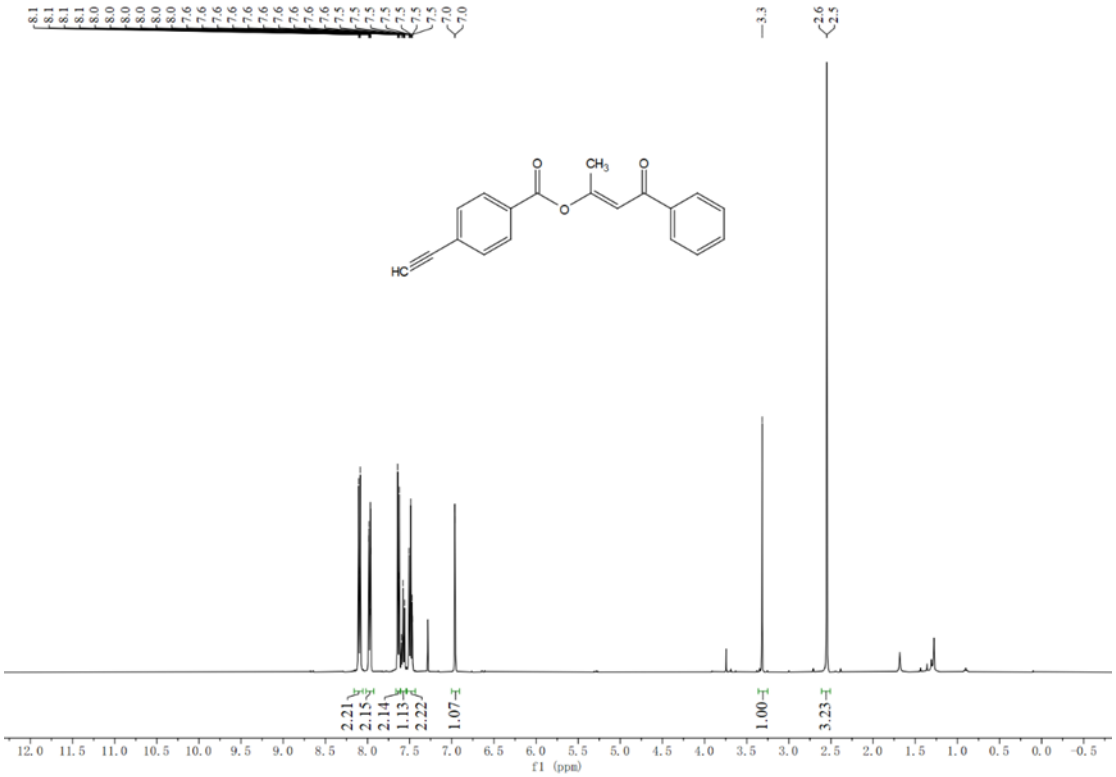


30 ^{1}H NMR

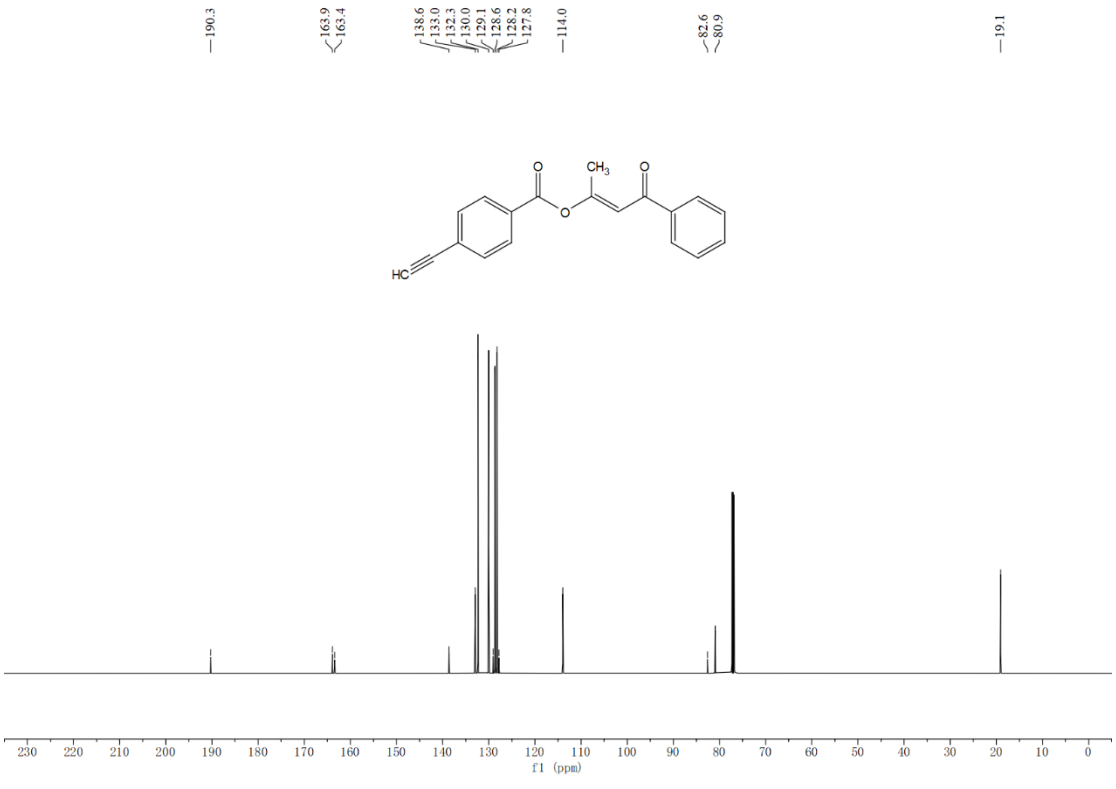


30 ^{13}C NMR

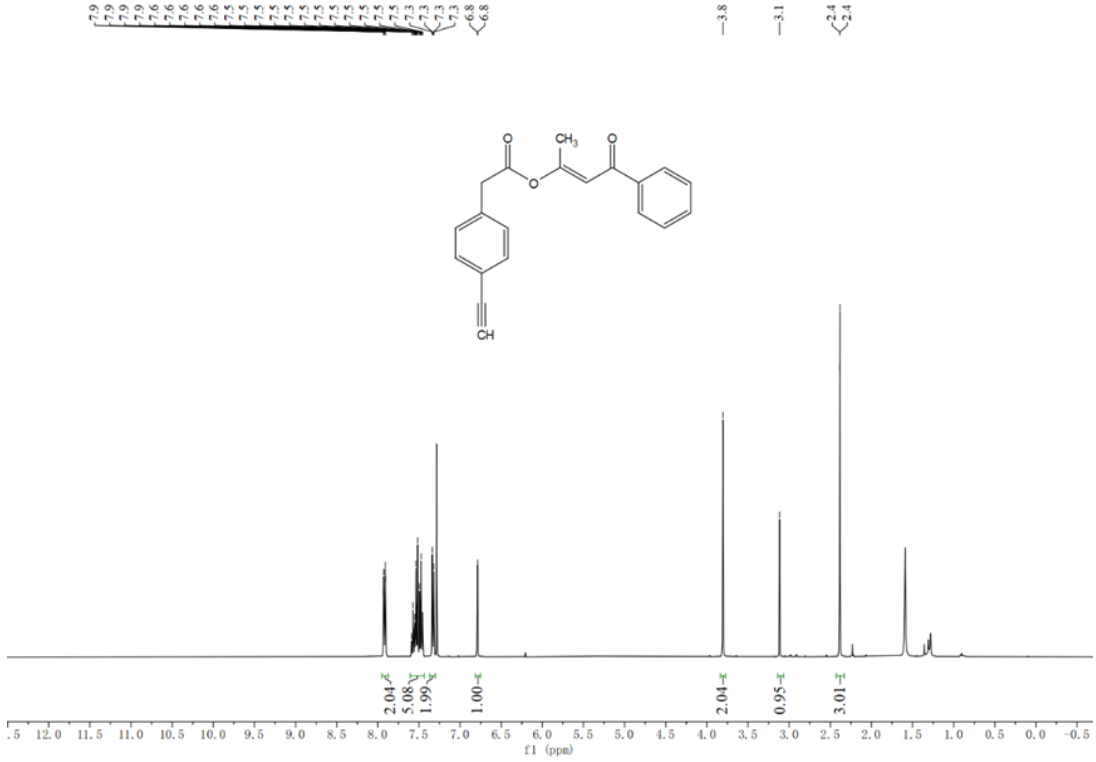
31 ^{1}H NMR



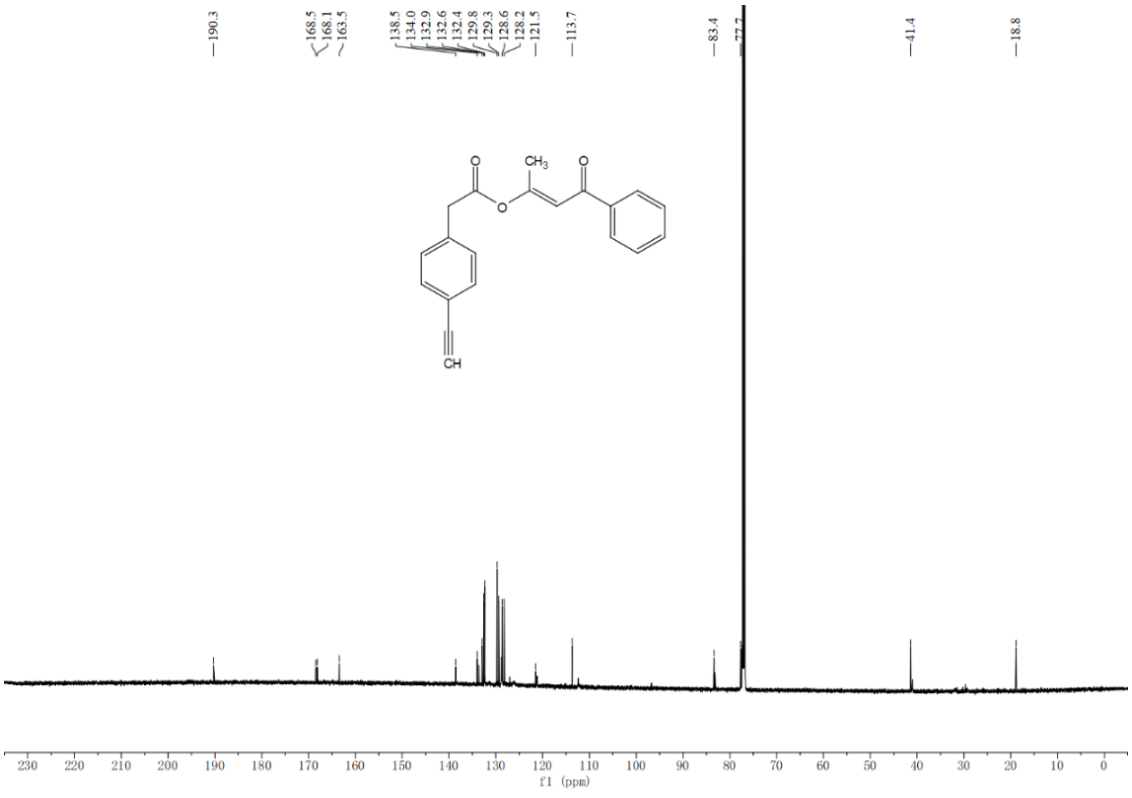
31 ^{13}C NMR



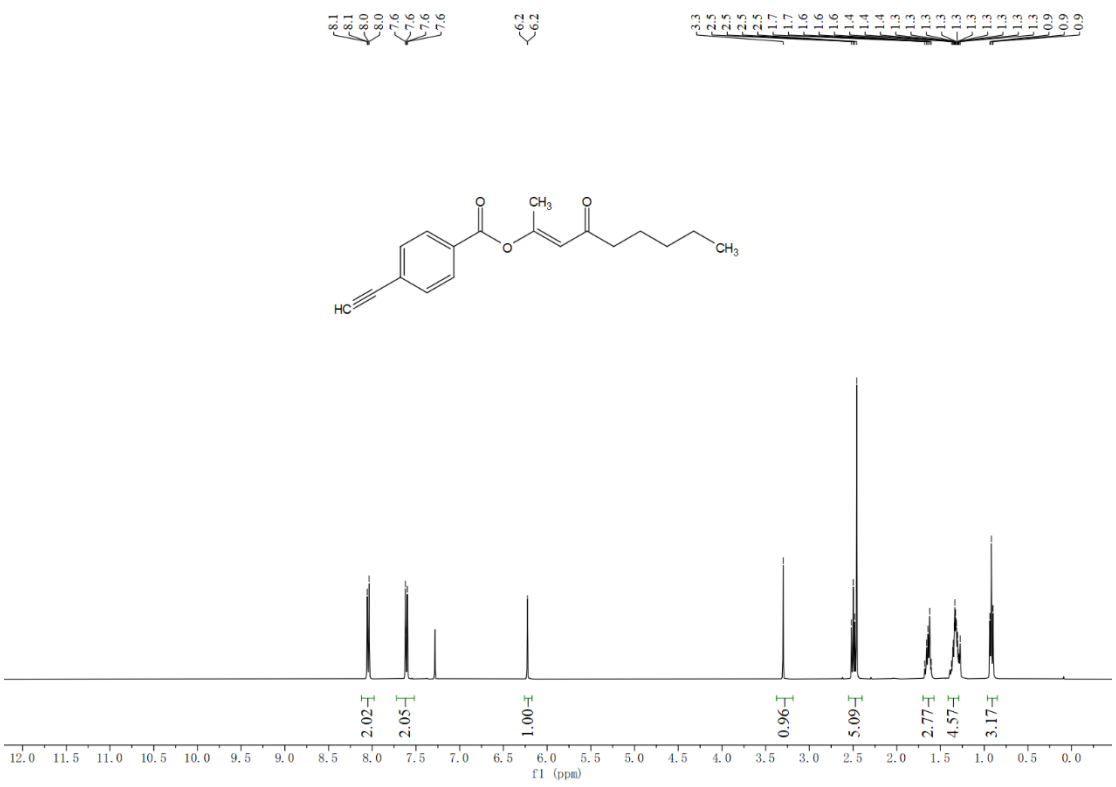
32 ^{1}H NMR



32 ^{13}C NMR

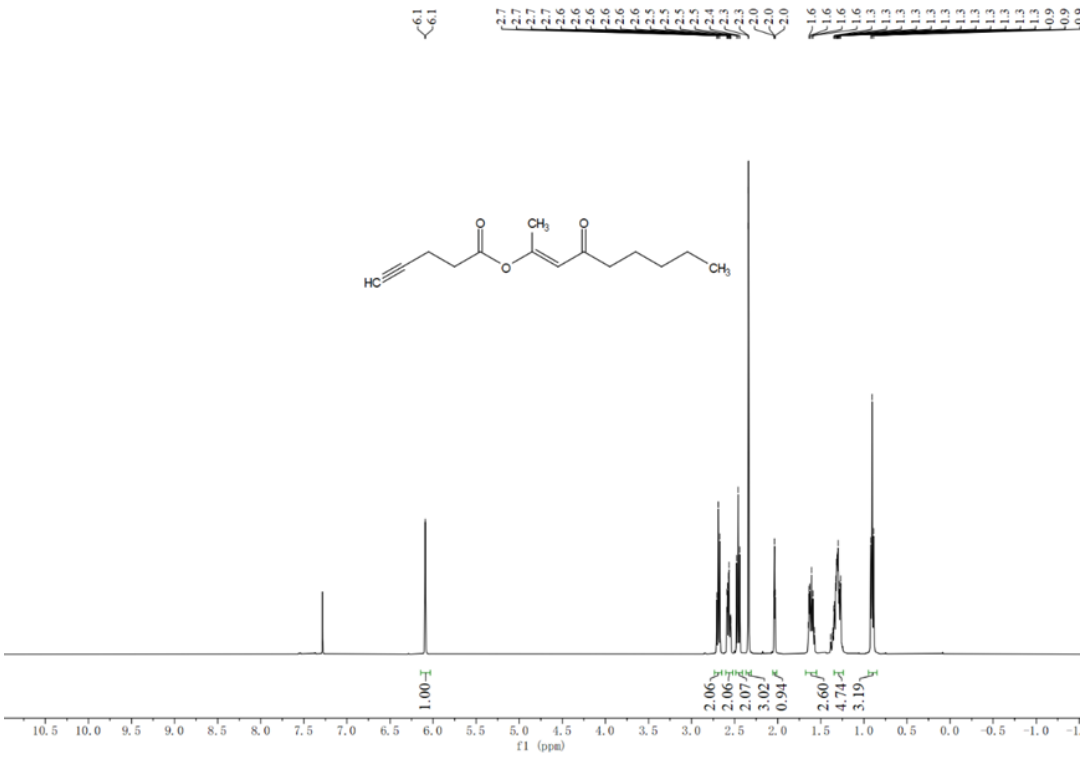


33 ^{1}H NMR

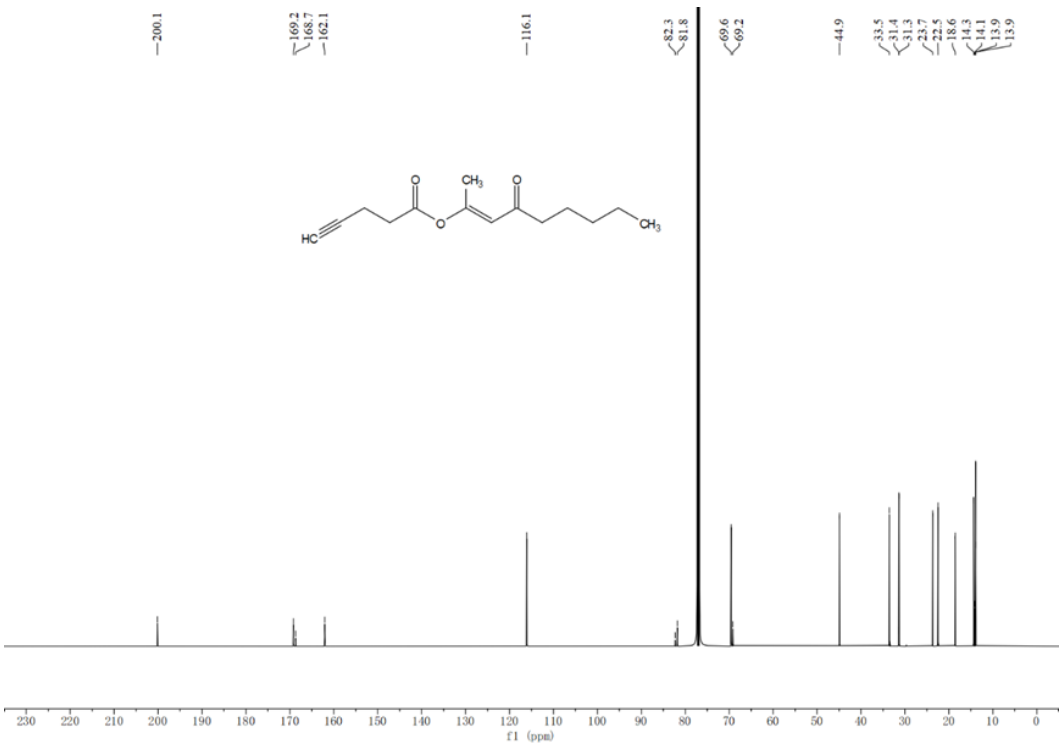


33 ^{1}H NMR

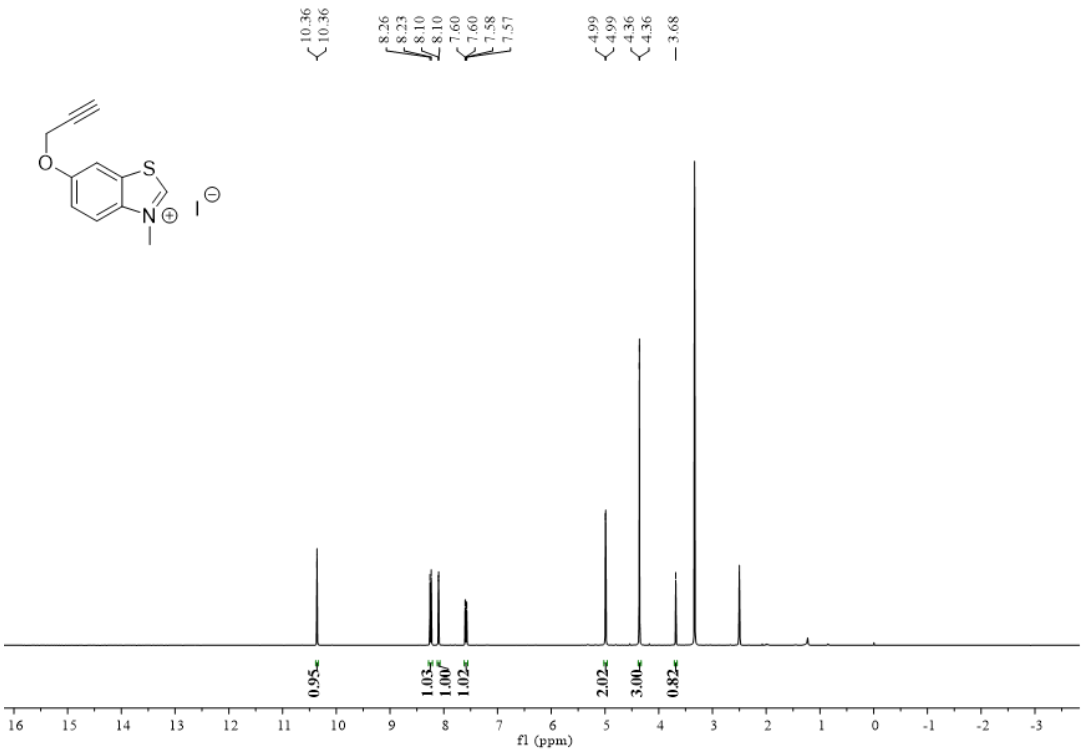
34 ^{1}H NMR



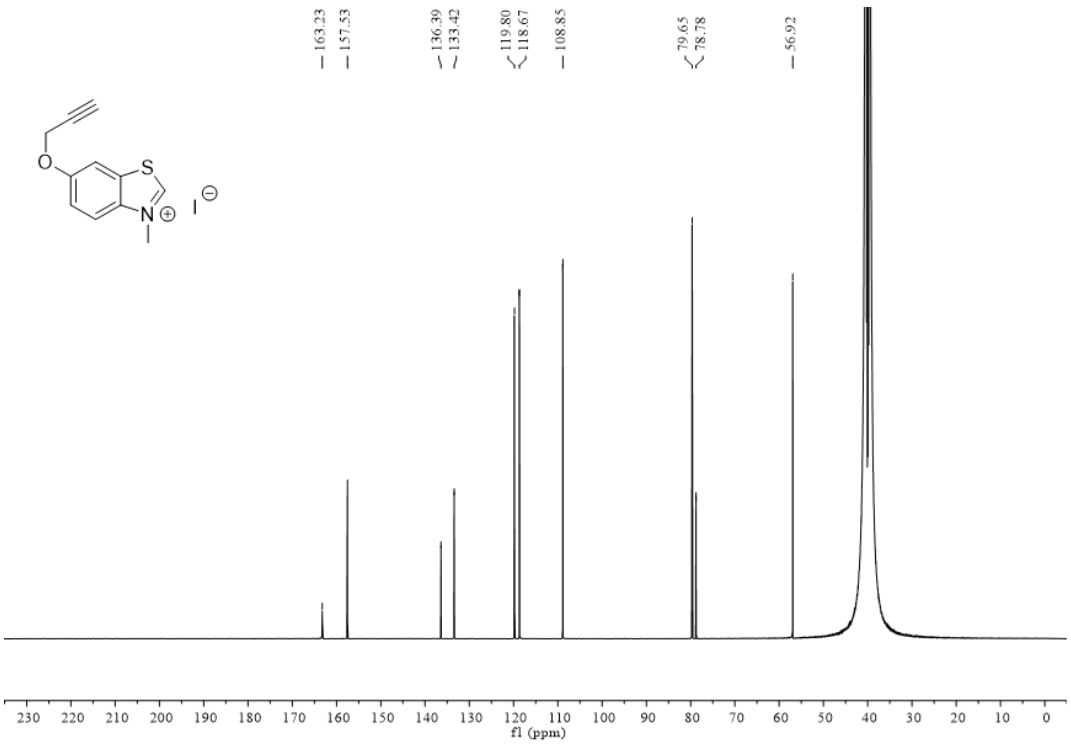
34 ^{13}C NMR



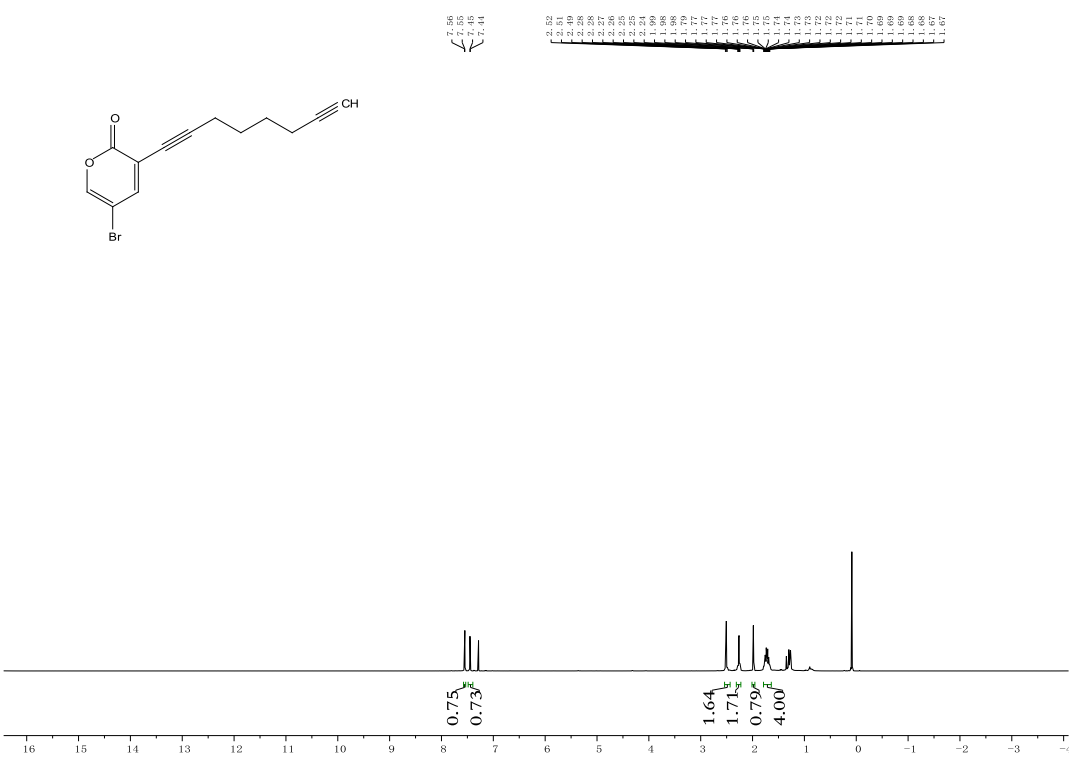
35 ^{1}H NMR



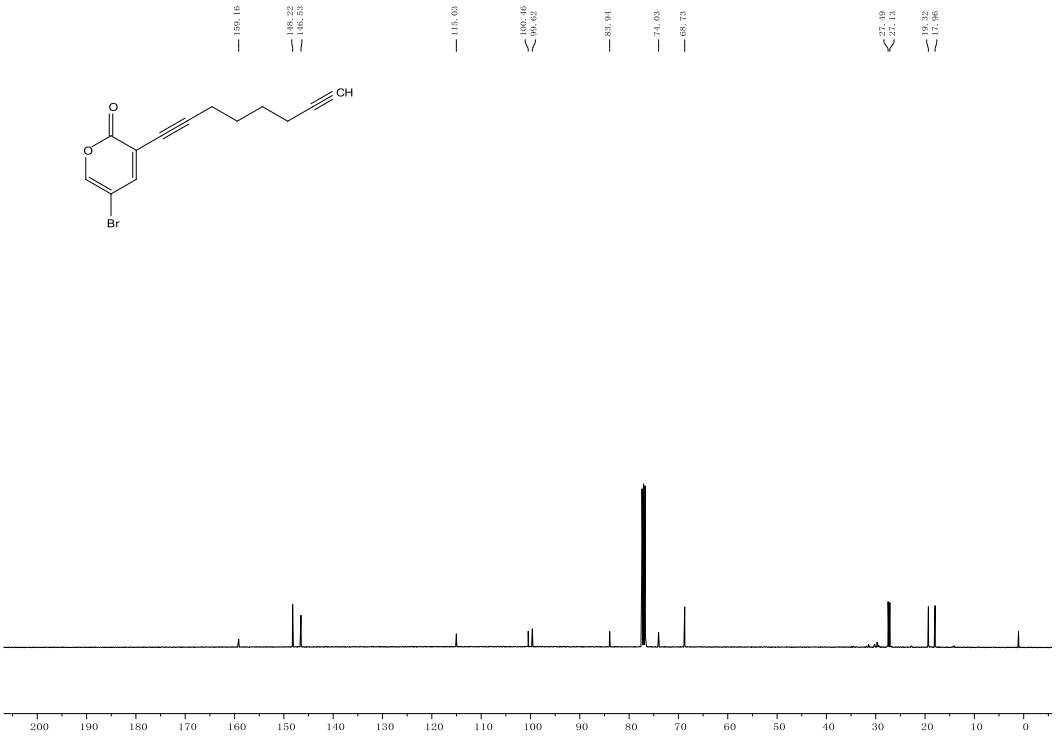
35 ^{13}C NMR



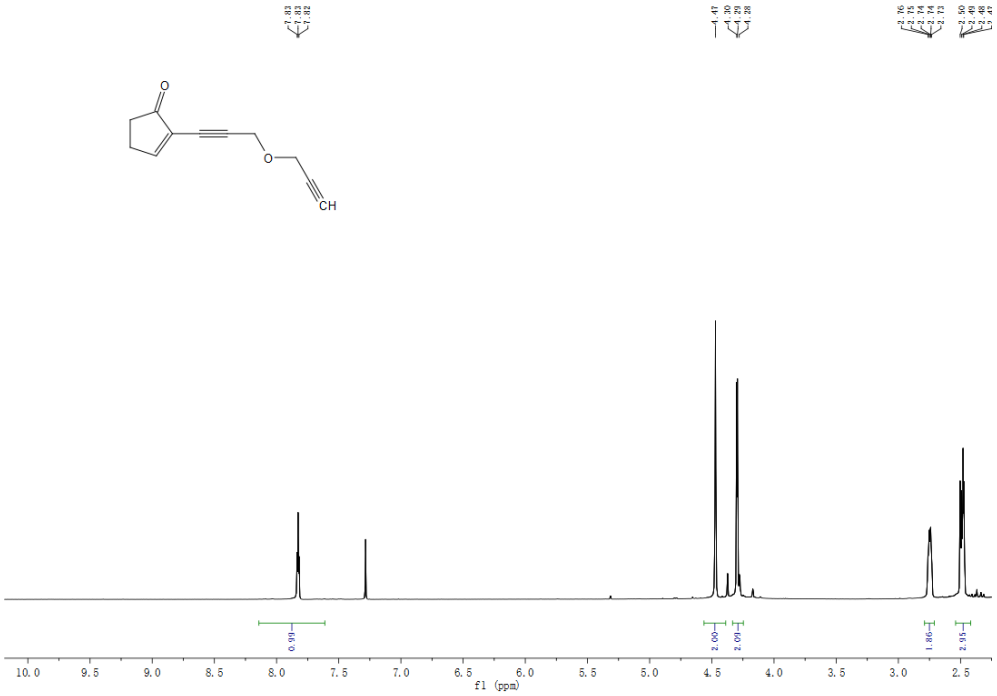
36 ^{1}H NMR



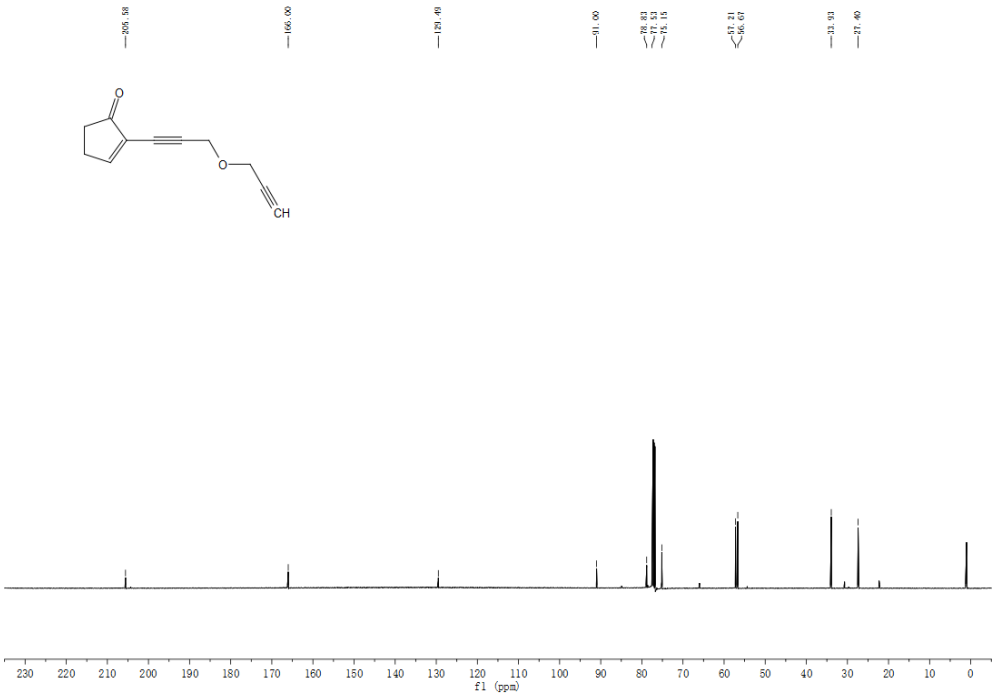
36 ^{13}C NMR



37 ^{1}H NMR

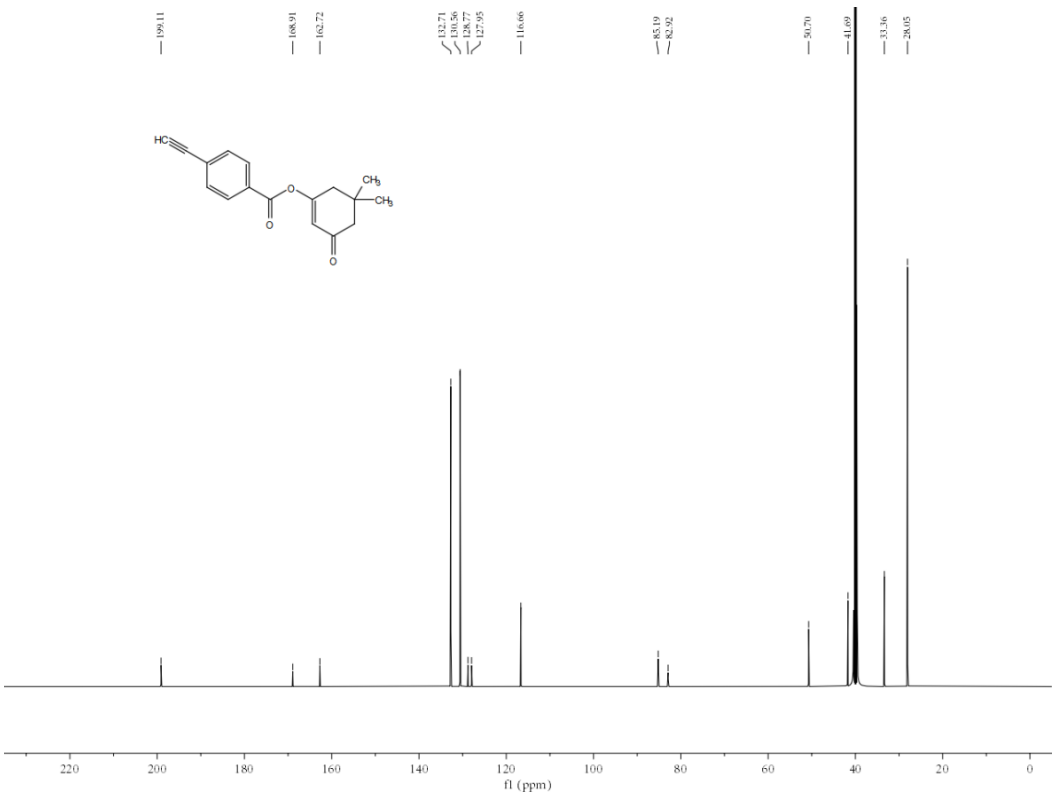


37 ^{13}C NMR

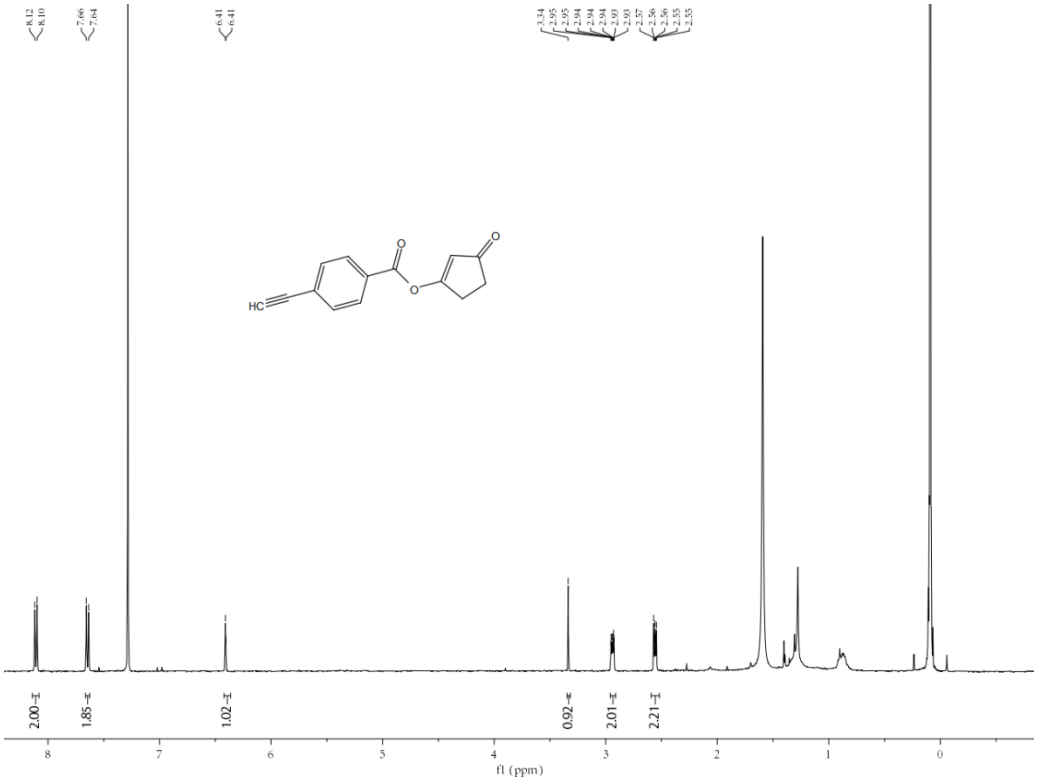


38 ^{1}H NMR

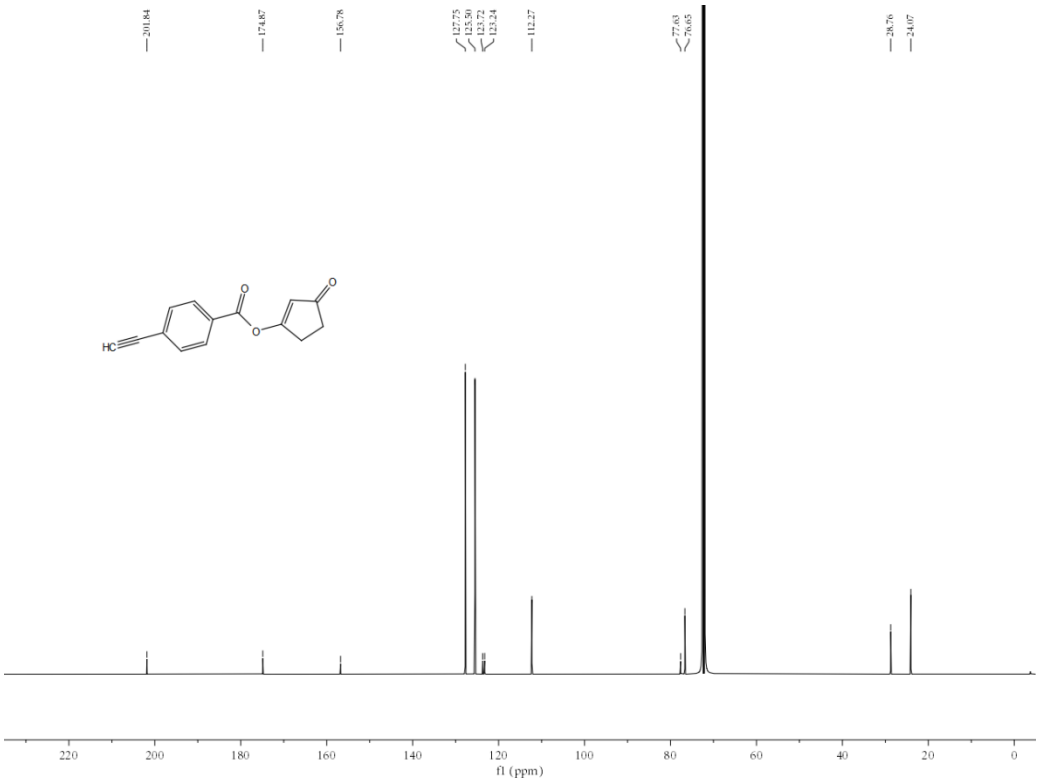
38 ^{13}C NMR



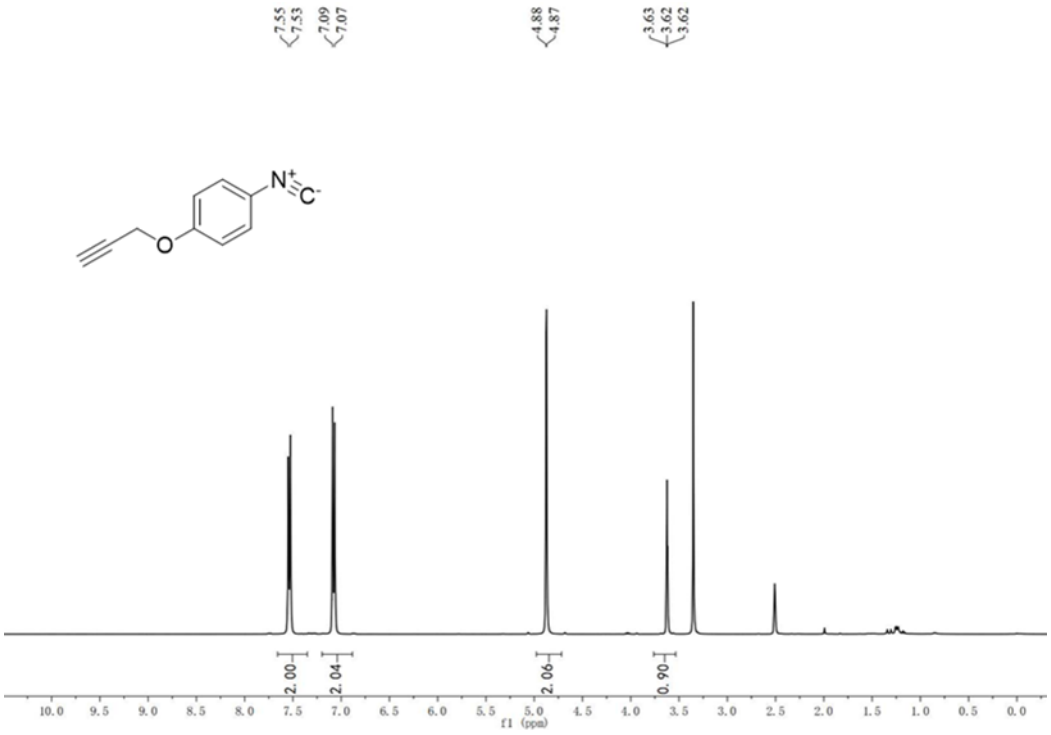
39 ^{1}H NMR



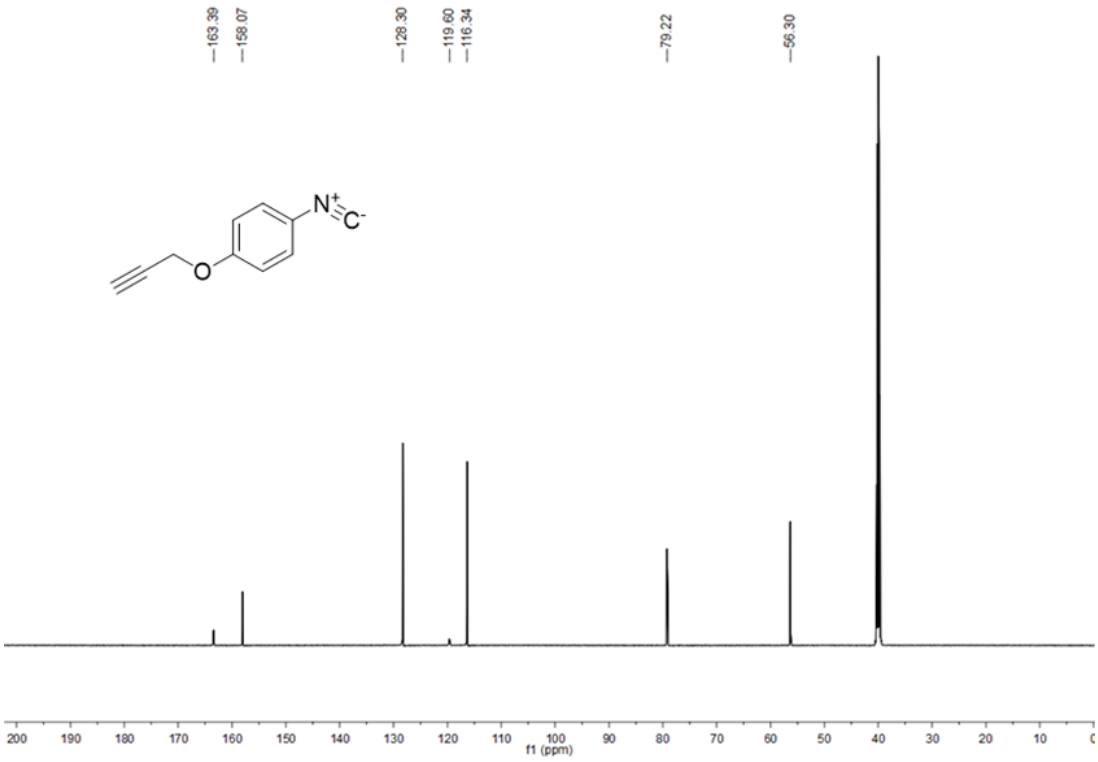
39 ^{13}C NMR



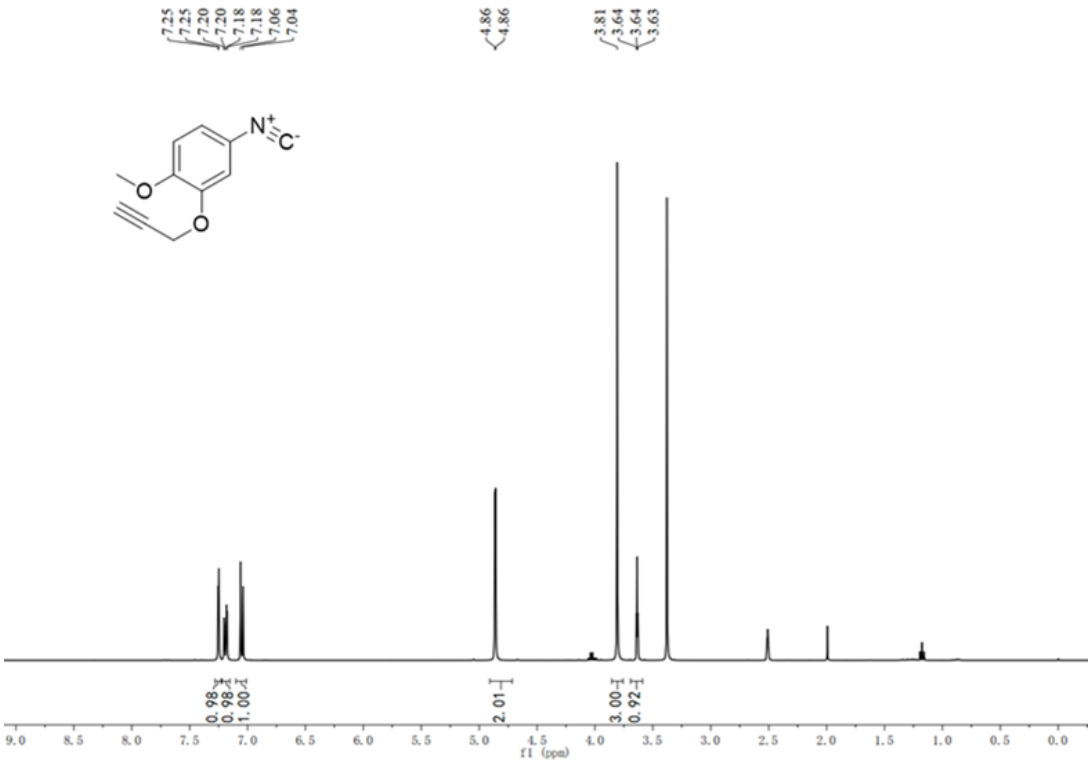
40 ^{1}H NMR



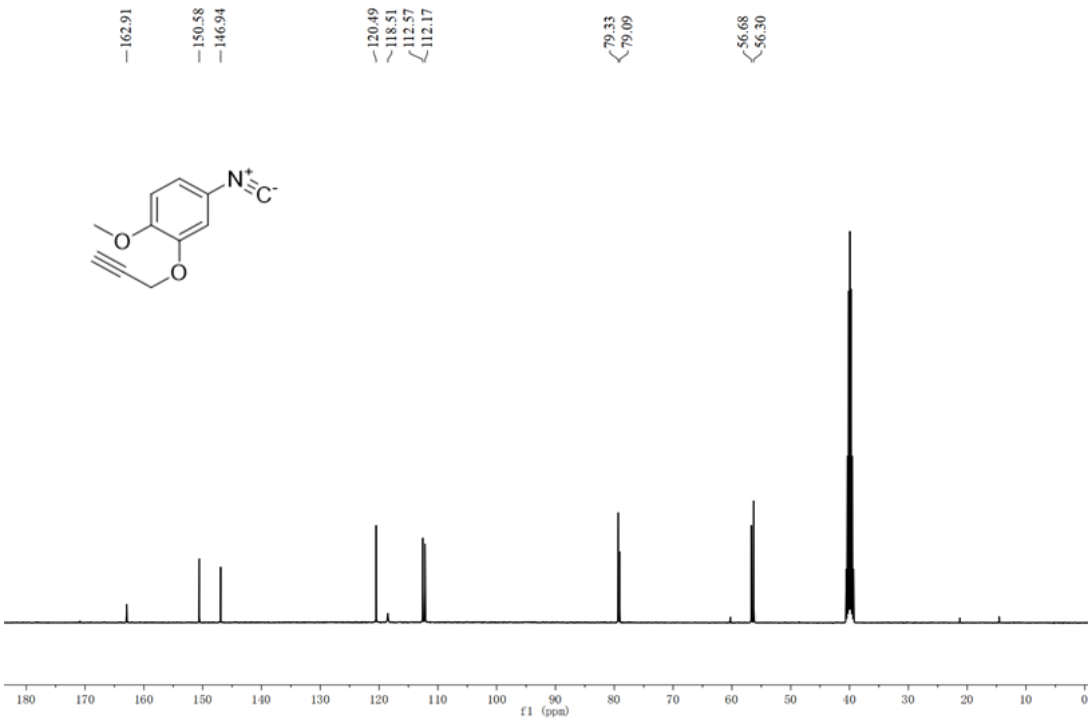
40 ^{13}C NMR



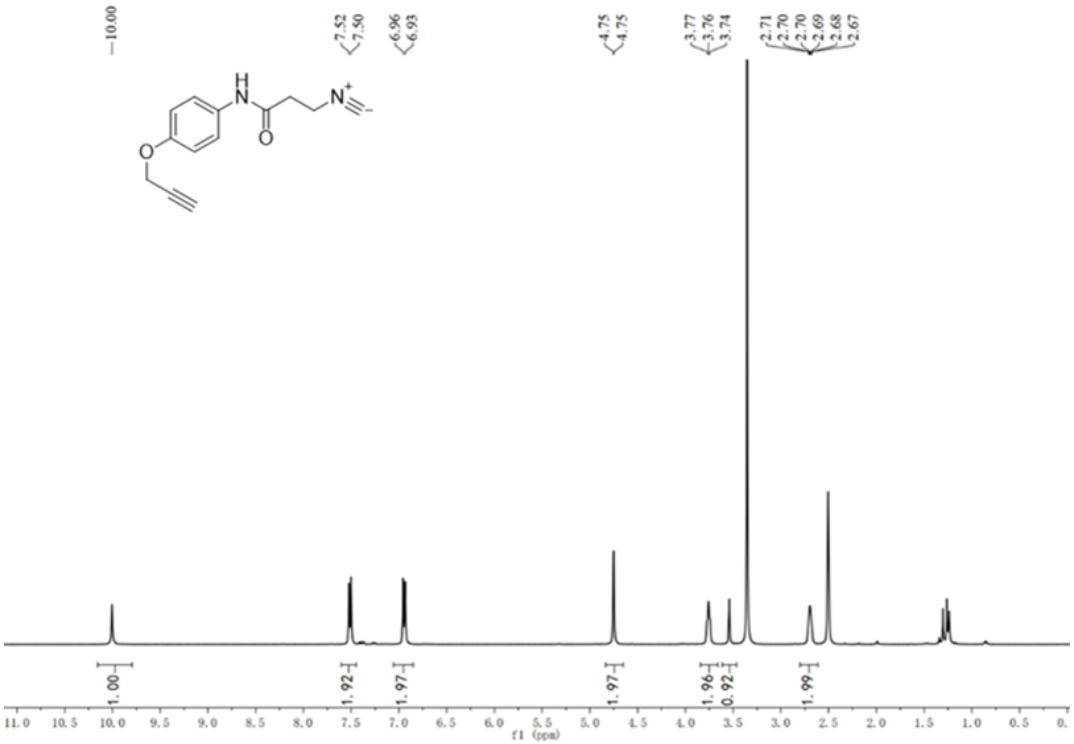
41 ^{1}H NMR



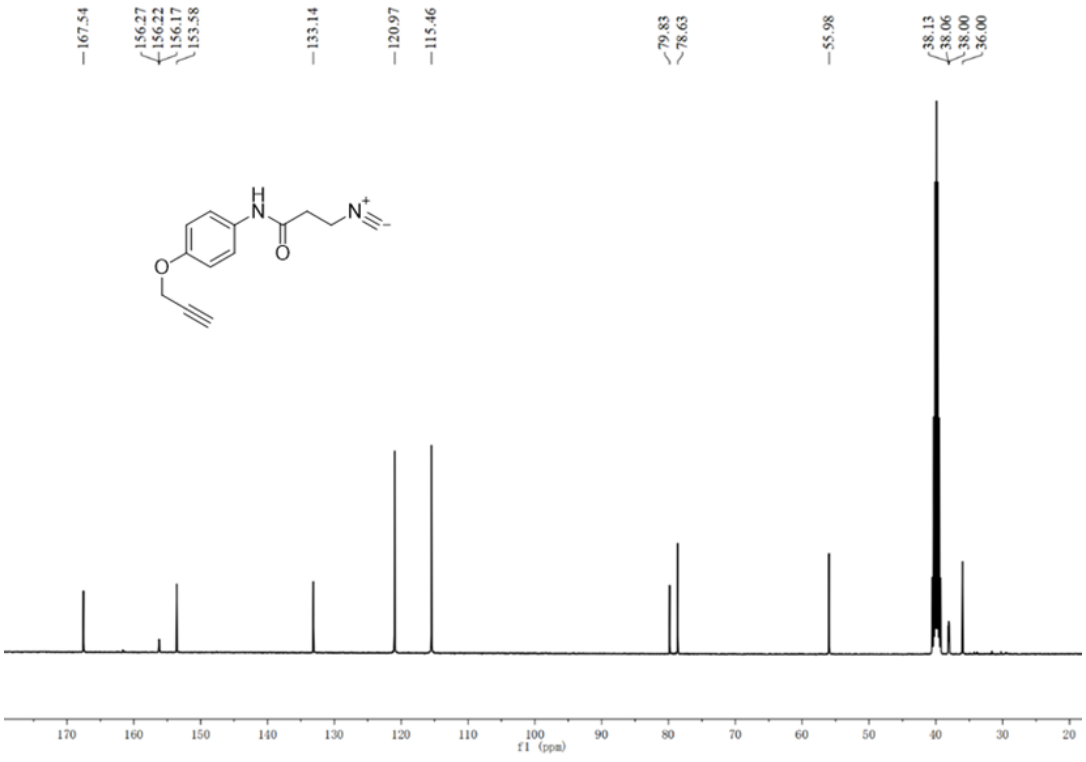
41 ^{13}C NMR



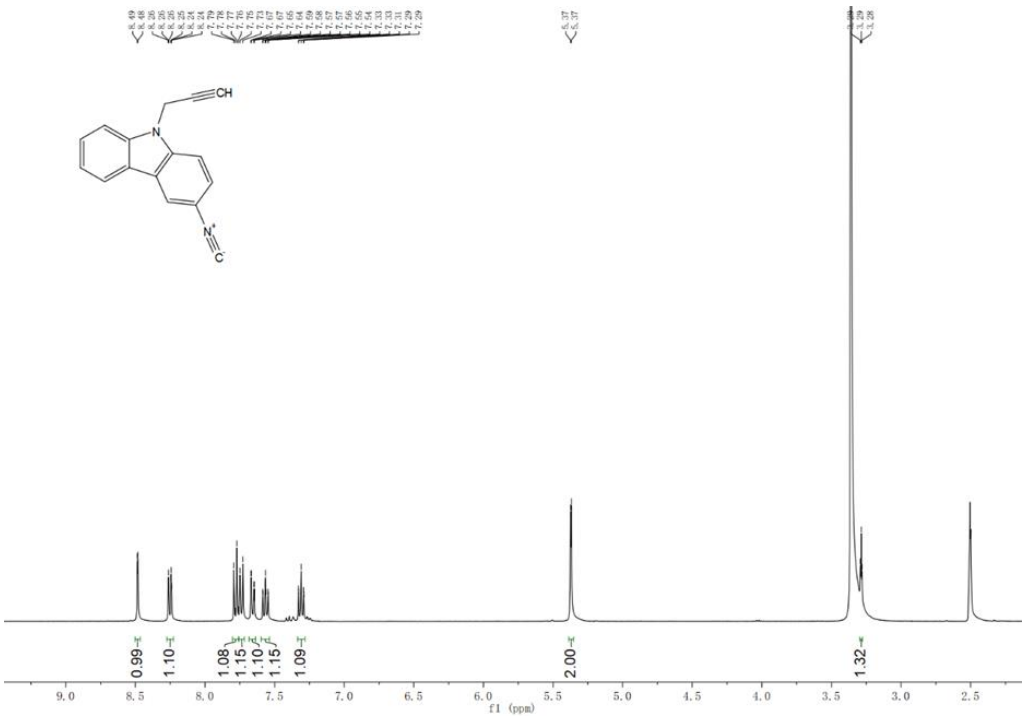
42 ^{1}H NMR



42 ^{13}C NMR



43 ^{1}H NMR



43 ^{13}C NMR