Electronic Supplementary Material (ESI) for ChemComm.


# Supporting Information

# A Highly Efficient Oxidative Condensation Reaction for Selective Protein Conjugation

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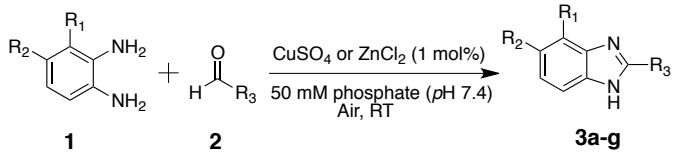
## General Method and Materials

Unless otherwise noted, all chemicals were purchased from reputable commercial sources (Sigma-Aldrich, Acros Organics, Alfa Aesar or TCI America) and used without further purification. Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). PCR products and products of restriction digestion were purified by gel electrophoresis and extracted using the Syd Labs Gel Extraction kit (Malden, MA). Plasmid DNA was purified using the Syd Labs Miniprep kit. DNA sequence analysis was performed by the Genomics Core at the University of California, Riverside (UCR). Mass spectrometry was performed with an Agilent ESI-TOF at the Analytical Chemistry Instrumentation Facility of UCR. $^{1}H$ and $^{13}C$ NMR spectra were recorded with a Varian Inova 400 MHz NMR spectrometer at the UCR Chemistry NMR Facility.

## General Synthetic Procedures

**Benzimidazoles (3a-3g):** Aryl diamine (0.1 mmol) and alkyl aldehyde (0.2 mmol) were mixed in 10 mL phosphate buffer (50 mM, pH 7.4) followed by addition of 20 μL of 50 mM $CuSO_4$ or $ZnCl_2$ aqueous solution at room temperature. The resulting mixture was stirred for the indicated time period (see Table 1). Next, $CH_2Cl_2$ (20 mL) was added to extract the crude product. The organic layer was obtained. The aqueous layer was re-extracted twice with $CH_2Cl_2$ (20 mL each). The combined organic layer was washed with $H_2O$ (20 mL), dried over anhydrous $Na_2SO_4$, and evaporated on a rotovap. The crude product was next purified by silica column chromatography (Hexane:EtOAc = 1:2).




**3b: 2-propyl-1*H*-benzo[*d*]imidazole**
$^{1}H$ NMR (400 MHz, $CDCl_3$) δ 7.53 (dd, J1=2.8 Hz, J2=5.6 Hz, 2H), 7.19 (dd, J1=2.8 Hz, J2=5.6 Hz, 2H), 2.9 (t, J=7.6 Hz, 2H), 1.87 (m, 2H), 0.97 (t, J=6.8 Hz, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 155.45, 138.61, 122.38, 114.79, 31.40, 21.88, 14.05; HR ESI-MS $[M+H]^+$ calcd: 161.1073 found: 161.1074.

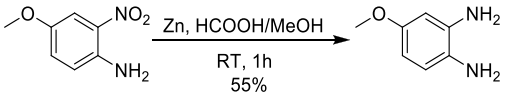
**3c: 4-methyl-2-propyl-1*H*-benzo[*d*]imidazole**
$^{1}H$ NMR (400 MHz, $CDCl_3$) δ 7.35 (d, J=7.6 Hz, 1H), 7.1 (t, J=7.2 Hz, 1H), 7.0 (d, J=7.6 Hz, 1H), 2.9 (t, J=7.6 Hz, 2H), 2.54 (s, 3H), 1.84 (m, 2H), 0.94 (t, J=7.2 Hz, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 154.9, 138.14, 124.92, 122.99, 122.4, 112.18, 104.97, 31.4, 22.08, 17.36, 14.05. HR ESI-MS $[M+H]^+$ calcd: 175.1230 found: 175.1230.

**3d: 5-methoxy-2-propyl-1*H*-benzo[*d*]imidazole**
$^{1}H$ NMR (400 MHz, $CDCl_3$) δ 7.4(d, J=8.4 Hz, 1H) 6.99 (d, J=2.4 Hz, 1H), 6.83 (dd, J1=2.4 Hz, J2=8.4 Hz, 1H), 3.78 (s, 3H), 2.85 (t, J=7.6 Hz, 2H), 1.84 (m, 2H), 0.96 (t, J=7.2 Hz, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 156.36, 155.04, 138.9, 133.45, 115.46, 111.59, 97.88, 56.07, 31.43, 21.88, 14.05. HR ESI-MS $[M+H]^+$ calcd: 191.1179 found: 191.1173.

**3e: methyl 2-propyl-1*H*-benzo[*d*]imidazole-6-carboxylate**
$^{1}H$ NMR (400 MHz, $CDCl_3$) δ 8.25 (s, 1H), 7.93 (d, J=8.4 Hz, 1H), 7.53 (d, J=8.4 Hz, 1H), 3.9 (s, 3H), 2.92 (t, J=7.6 Hz, 2H), 1.88 (m, 2H), 0.97 (t, J=7.2 Hz, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 168.03, 157.96, 142.32, 138.15, 124.39, 124.1, 116.92, 114.54, 52.32, 31.48, 21.72, 14.03. HR ESI-MS $[M+H]^+$ calcd: 219.1128 found: 219.1160.

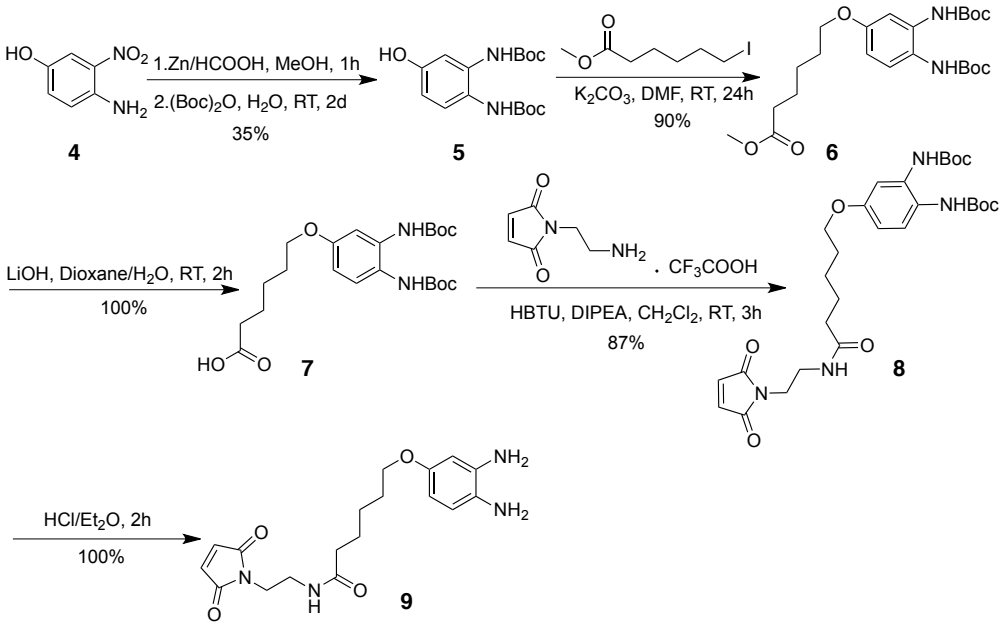
**3g: 2-cyclopropyl-4-methyl-1*H*-benzo[*d*]imidazole**
$^{1}H$ NMR (400 MHz, $CDCl_3$) δ 7.32 (d, J=8.4 Hz, 1H), 7.08 (t, J=7.6 Hz, 1H), 6.97 (d, J=7.6 Hz, 1H), 2.52 (s, 3H), 2.13 (m, 1H), 1.17 (m, 2H), 1.04 (m, 2H); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ156.36, 138.64, 137.47, 124.02, 122.93, 122.35, 112.5, 17.28, 10.01, 8.81. HR ESI-MS $[M+H]^+$ calcd: 173.1073 found: 173.1088.

**4-Methoxylbenzene-1,2-diamine (1d).** 4-Methoxylbenzene-1,2-diamine was synthesized in the laboratory from commercially available reagents. MeOH (10 mL) and HCl-activated Zn dust (700mg, 10.7 mmol) were added into a flask sitting in an ice bath. Next, 4-methoxy-2-nitroaniline (336 mg, 2 mmol) was added. HCOOH (1 mL) was slowly introduced into the flask. The resulting mixture was warmed up to room temperature and stirred for 1 h before filtration. The filtrated was concentrated by rotovap and purified on silica chromatography column (EtOAc/MeOH = 20:1) to afford the product as brown solid (152 mg, 1.1 mmol). The overall yield was 55%.




$^1$H NMR (400 MHz, $CDCl_3$) δ 6.63 (d, J=8.4 Hz, 1H) 6.3 (d, J=2.8 Hz, 1H), 6.24 (dd, J1=2.8 Hz, J2=8.4 Hz, 1H), 3.71 (s, 3H), 3.33 (br, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 154.93, 137.26, 127.27, 118.68, 104.39, 103.24, 55.75. ESI-MS $[M+H]^+$ calcd: 139.08 found: 139.08.

**Scheme S1. Synthetic route to the aryl diamine maleimide linker**




**di-*tert*-butyl 4-hydroxy-1,2-phenylenedicarbamate (5).** To the suspension of HCl-activated Zn dust (700mg, 10.7 mmol) in $CH_3OH$ (5 mL) in ice-bath, added was 4-amino-3-nitrophenol (**4**, 308 mg, 2 mmol). Next, HCOOH (1 mL) was introduced. The resulting mixture was stirred for 1 h before filtration. The filtrate was concentrated to afford a dark brown oilish residue, which was used directly without purification. The residue was dissolved in $CH_3OH$ (10 mL) followed by addition of $(Boc)_2O$ (4 mmol, 890 mg). The solution was allowed to stir at RT for 2 d, and then extracted with $CH_2Cl_2$ (15 mL) for three times. The combined organic layer was dried over anhydrous $Na_2SO_4$ and concentrated in vacuo. The residue was purified through silica chromatography (EtOAc/Hexane = 1:2) to yield *t*-butyl 4-hydroxy-1,2-phenylenedicarbamate as light pink solid (228.9 mg, 0.706 mmol). The overall yield was 35%.

$^1$H NMR (400 MHz, d6-acetone) δ 8.24 (br, 1H), 7.75 (br, 1H), 7.25 (s, 1H), 7.12 (d, J=8.4Hz, 1H), 6.52 (dd, J1=8.4Hz, J2=2.4Hz, 1H), 1.51 (s, 9H), 1.5 (s, 9H); $^{13}$C NMR (100 MHz, d6-acetone) δ 155.37, 154.61, 153.28, 133.83, 126.81, 121.27. 110.81, 109.29, 79.62, 79.37, 28.61, 27.81. ESI-MS $[M+Na]^+$ calcd: 347.16 found: 347.16.

**6-Iodo-methylhexonate.** 6-Bromo-methylhexonate (1 mmol, 160.8 μL) and NaI (2 mmol, 300 mg) were added into acetone (15 mL) at room temperature. The mixture was next refluxed for 3 h. Acetone was removed by rotovap and the residue was dissolved in $CH_2Cl_2$ and washed with $H_2O$. The organic layer was dried over

anhydrous $Na_2SO_4$ and concentrated by rotovap to afford 6-iodo-methylhexonate (240.7 mg) as colorless oil, which was next used directly without purification.

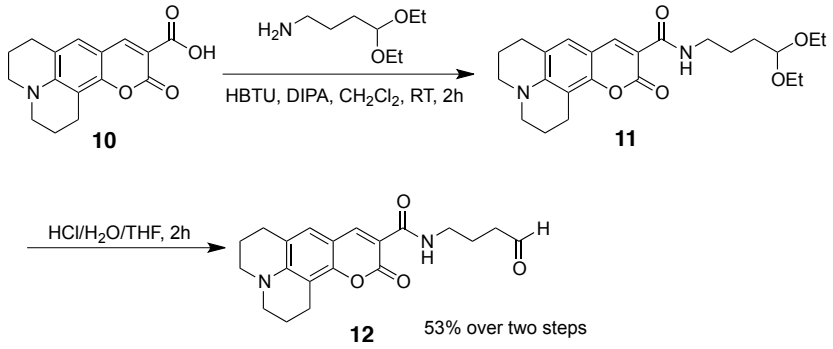
**Methyl 6-(3,4-bis(*tert*-butoxycarbonylamino)phenoxy)hexanoate (6).** 6-iodo-methylhexonate (240.7 mg, 0.96 mmol) and **5** (228.9 mg, 0.706 mmol) were dissolved in DMF (10 mL) at room temperature. Next, $K_2CO_3$ (276 mg, 2 mmol) was added. The reaction was continued for 24 h before removal of the solvent. The residue was dissolved in $CH_2Cl_2$ (20 mL) and washed with $H_2O$ (20 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated to yield a yellow oil, which was further purified through silica column chromatography (EtOAc/Hexane = 1:4) to give pure **6** (287.3 mg, 0.63 mmol). The yield was 90%.
$^1$H NMR (400 MHz, $CDCl_3$) δ 7.12 (br, 1H), 6.9 (br, 1H), 6.54 (dd, J1=8.4Hz, J2=2.4Hz, 1H), 3.89 (t, J=6.4Hz, 2H), 3.63 (s, 3H), 2.8 (t, J=7.2Hz, 2H), 1.61-1.78 (m, 6H), 1.47 (s, 9H), 1.46 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.28, 157.68, 154.78, 153.4, 133.8, 127.07, 121.23, 110.9, 108.56, 81.14, 80.88, 68.05, 51.68, 34.17, 29.09, 28.48, 27.95, 25.83, 24.88. ESI-MS $[M+Na]^+$ calcd: 475.25 found: 475.25.

**6-(3,4-Bis(*tert*-butoxycarbonylamino)phenoxy)hexanoic acid (7).** **6** (86 mg, 0.19 mmol) and LiOH (31 mg, 0.75 mmol) were added into a dioxane/$H_2O$ mixture (3:1, 2.4 mL) in ice-bath. The solution was stirred for 2 h. Then the solution was acidified with 1 M HCl and extracted with EtOAc (3x 20 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated to afford **7** as oil, which was used directly without purification. The yield was 100%.
$^1$H NMR (400 MHz, $CDCl_3$) δ 7.18 (br, 1H), 6.94 (br, 1H), 6.56 (d, J=8.4Hz, 1H), 3.91 (t, J=6.4Hz, 2H), 2.35 (t, J=7.2Hz, 2H), 1.63-1.77 (m, 6H), 1.49 (s, 9H), 1.48 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 179.29, 157.72, 154.97, 153.72, 137.06, 128.58, 121.19, 110.97, 108.64, 81.1, 80.94, 68.05, 34.12, 29.9, 29.64,29.43, 25.4, 24.6. ESI-MS $[M-H]^-$ calcd: 437.22 found 437.22

**di-*tert*-butyl (4-((6-((2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)amino)-6-oxohexyl)oxy)-1,2-phenylene)dicarbamate (8).** **7** (44.7 mg, 0.102 mmol) was dissolved in $CH_2Cl_2$ (5 ml). Then HBTU (38.7 mg, 0.102 mmol) was added followed by addition of DIPEA(17.7 µl, 0.102 mmol). The solution was stirred at 0°C for 30 min before *N*-(2-aminoethyl)-maleimide trifluoromethylacetate (26.7 mg, 0.105 mmol) and DIPEA(18 µl, 0.105 mmol) were added. The mixture was stirred for another 3 h before it was acidified with diluted HCl. The organic layer was separated and the water phase was extracted twice with $CH_2Cl_2$ (10 mL each). The combined organic layer was dried over anhydrous $Na_2SO_4$ and concentrated by rotovap. The residue was purified through silica chromatography (EtOAc/Hexane = 2:1) to afford an oil product (50 mg, 0.089 mmol). The yield was 87%.
$^1$H NMR (400 MHz, $CDCl_3$) δ 7.1 (br, 1H), 6.93 (br, 1H), 6.55 (s, 2H), 6.01 (dd, J1=8.4Hz, J2=2.4 Hz, 1H), 3.89 (t, J=6.4Hz, 2H), 3.63 (t, J=6Hz, 2H), 3.40 (m, 2H), 2.11 (t, J=7.2Hz, 2H), 1.58-1.75 (m, 6H), 1.47 (s, 9H), 1.46 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 173.76, 171.14, 157.68, 154.82, 153.47, 134.43, 127.01, 126.95, 111.16, 110.94, 108.7, 80.96, 80.85, 68.08, 53.63, 38.96, 37.72, 36.51, 29, 28.49, 25.83, 25.33. HR ESI-MS $[M+H]^+$ calcd: 561.2919 found: 561.2911; $[M+Na]^+$ calcd: 583.2738 found: 583.2683.

**6-(3,4-diaminophenoxy)-*N*-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)hexanamide (9).** **8** (10 mg, 0.018 mmol) was added to a TFA (1 mL) and $CH_2Cl_2$(1 mL) mixture. The solution was stirred at room temperature for 30 min, before the solvent was removed by rotovap to afford a white solid **9** (6.4 mg, quantitative). HR ESI-MS $[M+H]^+$ calcd: 361.1870 found: 361.1873.

**Scheme S2. Synthetic Route to the Coumarin 343 aldehyde dye**




To Coumarin 343 (**10**, 15 mg, 0.05 mmol) in $CH_2Cl_2$ (3 mL) was added HBTU (20 mg, 0.052 mmol) and DIPEA(9.1 μL, 0.052 mmol) in ice-bath. The solution was stirred for 30 min. Next, 4-aminobutyraldehyde diethylacetal (9 uL, 0.05 mmol) in $CH_2Cl_2$ (1 mL) was introduced. The solution was stirred for another 2 h before it was sequentially washed with diluted HCl (1 M, 20 mL), saturated $NaHCO_3$ solution (20 mL) and $H_2O$ (20 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated by rotovap. The residue (**11**) was dissolved in THF/$H_2O$ (3:1, 4 mL) followed by addition of concentrated HCl (100 μL) at room temperature. The solution was stirred for another 2 h. The resulting solution was neutralized with saturated $NaHCO_3$ solution and extracted three times with $CH_2Cl_2$ (20 mL each). The combined organic layer was dried over anhydrous $Na_2SO_4$ and concentrated by rotovap. The crude product was purified by silica chromatography (EtOAc/Hexane = 2:1, containing 0.5% TEA) to yield the product as yellow solid (9.4 mg, 0.027 mmol). The yield was 53% over two steps. $^1$H NMR (400 MHz, $CDCl_3$) δ 9.78 (s, 1H), 8.9 (br, 1H), 8.57 (s, 1H), 6.98 (s, 1H), 3.46 (t, J=6 Hz, 2H), 3.3 (m, 4H), 2.87 (t, J=6.4Hz, 2H), 2.75 (t, J=6Hz, 2H), 2.55 (t, J=6.4Hz, 2H), 1.94 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 201.92, 164.07, 163.31, 152.88, 148.34, 127.24, 119.86, 109.01, 108.46, 105.88, 105, 50.44, 50.03, 41.54, 38.87, 27.68, 22.58, 21.34, 20.41, 20.32. HR ESI-MS $[M+H]^+$ calcd: 355.1652 found: 355.1672.

## Reaction Kinetics

To estimate the rate constant of the reaction between 4-methoxybenzene-1,2-diamine and butyraldehyde, 0.1 mmol 4-methoxybenzene-1,2-diamine and 0.15 mmol butyraldehyde were added into 100 mL phosphate buffer (50 mM, *p*H 7.4), followed by addition of $CuSO_4$ aqueous solution to a final concentration of 10 μM. The resulting mixture was stirred at room temperature for 50 min. In every 10 min, 10 mL reaction mixture was removed from the flask and quickly extracted into 2 mL $CH_2Cl_2$. The organic layer was treated with anhydrous $Na_2SO_4$. The $CH_2Cl_2$ solvent and excess butyraldehyde were removed by rotovap at room temperature. The processing steps were completed in less than 3 min to assure a timely termination of reaction. The crude residue was dissolved in $CDCl_3$ and used directly for $^1$H NMR analysis. A characteristic change in proton chemical shift of the methoxy group substituted in the aryl ring was observed. The areas of the corresponding peaks were integrated and utilized to estimate the molar concentrations of the product and reactants at various time points. A second-order reaction kinetics were utilized to fit the data of the product formation and derive the rate of the reaction as 1.61 $M^{-1}S^{-1}$.

## Gene Cloning, Plasmid Construction and Protein Purification

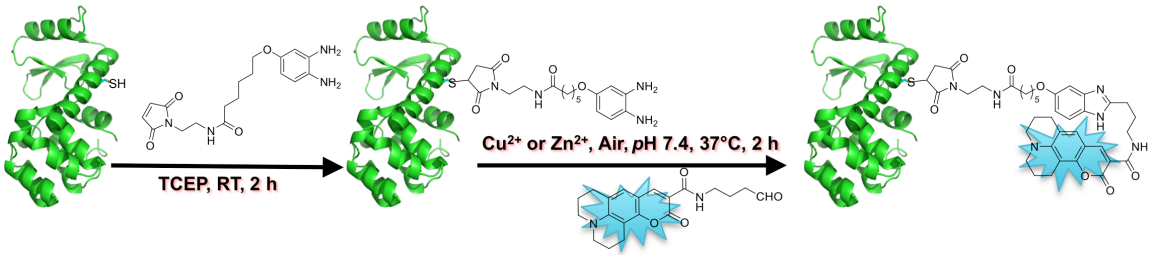
Oligonucleotides T4L_F (tagcaccatggatatatttgaaatg) and T4L68C_R (aacatcctggcaaaagagttt) were used to amplify a T4 lysozyme gene fragment containing no cysteine residues. In parallel, oligonucleotides T4L68C_F (aaactcttttgccaggatgtt) and T4L_R2 (ttcgctcgagttaatggtgatggtgatgatgacctaggttcttatacgcgtcccaagtgc) were used to amplify the same template. The two PCR fragments were joined together with the two primers T4L_F and T4L_R2 in an overlap PCR. The gene encoding T4 lysozyme contain a single cysteine at residue 68 were digested with Noc 1 and Xho 1 restriction endonucleases, and ligated into a pre-treated compatible pCDF-1b plasmid.

The expression plasmid was introduced into BL21(DE3) *E. coli* cells by electroporation. Cells were grown on LB agar plates overnight at 37°C to form colonies. A single colony was grown overnight in 5 ml LB media supplemented with spectinomycin (50 μg/ml) at 37°C. Saturated overnight culture was then diluted by 100x and grown to OD600 = 0.8. Cells were then induced with 1mM IPTG. After induction, *E. coli* was shaken at 25°C for another 14-16 hours. Cells were harvested by centrifugation and lysed with B-PER II protein extraction reagent (Pierce) supplemented with TCEP (1 mM). The 6xHis tagged protein was purified using Ni-NTA agarose beads (Qiagen) under native conditions according to the manufacturer's instructions. TCEP (1 mM) was added into the Lysis, Wash and Elution buffers to maintain the protein in a reduced state. The protein eluted from Ni-NTA beads was overnight dialyzed at 4°C into phosphate buffer (50 mM, *p*H 7.4) containing TCEP (1 mM).

## Protein Labeling

The total reaction volume was 100 μL. The T4L-N68C protein (50 μM) was mixed with the aryl diamine maleimide linker compound (**9**, 500 μM) in phosphate buffer (50 mM, *p*H 7.4) containing TCEP (1 mM). The mixture was incubated for 2 hours at room temperate. Micro Bio-Spin 6 Desalting Columns (Bio-Rad) were used to remove excess small molecules, and the resulting protein containing an installed aryl diamine functional group was directly used for reaction with the Coumarin aldehyde dye (**12**). The protein was diluted with phosphate buffer (50 mM, *p*H 7.4) to a concentration of 50 μM. **12** (200 μM) and $CuSO_4$ (2.5 μM) or $ZnCl_2$ (2.5 μM) were added. The mixture was incubated in open air at 37°C for 2 hours. The protein was directly used for SDS-PAGE characterization, or desalted using Micro Bio-Spin 6 Columns for fluorescence measurement, or precipitated with methanol/chloroform for mass spectrometry characterization. Mass spectra were recorded on an Agilent ESI-TOF instrument by direct infusion of proteins dissolved in formic acid/water (1:100) solution.

**Scheme S3. Route to label T4 lysozyme (T4L-N68C) in two sequential steps**




## Protein Sequence (T4L-N68C)

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MDIFEMLRID EGLRLKIYKD TEGYYTIGIG HLLTKSPSLN AAKSELDKAI GRNTNGVITK   60
DEAEKLFCQD VDAAVRGILR NAKLKPVYDS LDAVRRAALI NMVFQMGETG VAGFTNSLRM   120
LQQKRWDEAA VNLAKSRWYN QTPNRAKRVI TTFRTGTWDA YKNLGHHHHH H            171
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**Table S1. Some common bioconjugation reactions. The last 3 rows are a comparison of bioconjugation reactions involving aldehyde.**

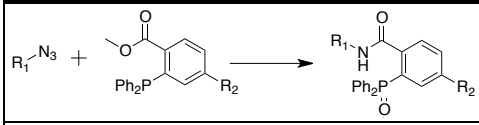
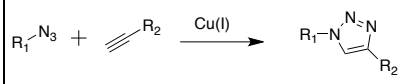
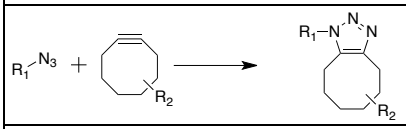
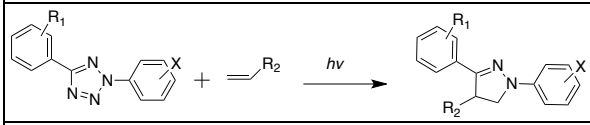
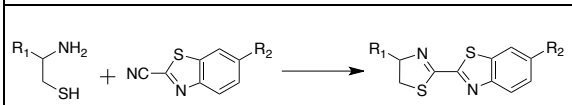
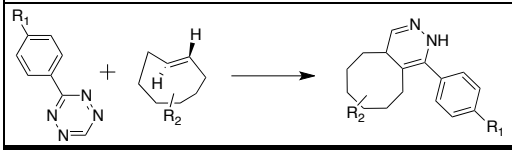
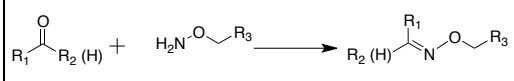
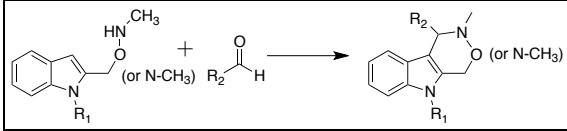
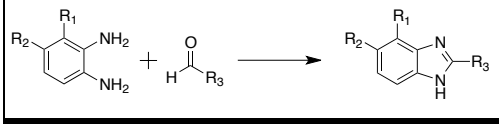
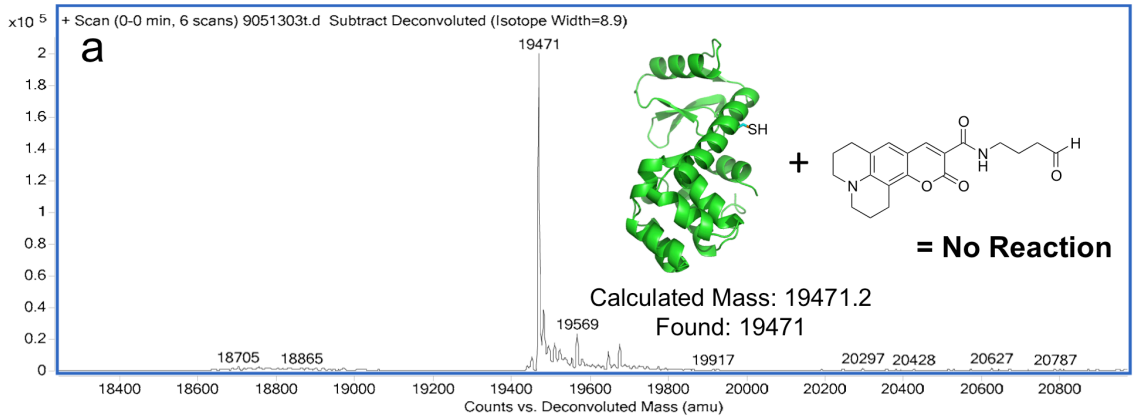
| Example Reactions | Notes |
|---|---|
|  | **Staudinger-Bertozzi ligation[1,2]**<br>• Rate constant: ~ $10^{-3}$ $M^{-1}s^{-1}$ |
|  | **Cu(I)-catalyzed azide–alkyne cycloaddition[2-4]**<br>• Rate constant: ~$10^{-1}$-10 $M^{-1}s^{-1}$<br>• Require Cu(I) (1 mM for optimal reaction); cell toxicity |
|  | **Strain-promoted azide–alkyne cycloaddition[2,5]**<br>• Rate constant: ~ $10^{-3}$-1 $M^{-1}s^{-1}$<br>• Challenging to prepare ring-strained cyclooctyne derivatives |
|  | **Tetrazole-alkene photoclick chemistry[6,7]**<br>• Rate constant: ~ 1 $M^{-1}s^{-1}$<br>• Rapidly inducible with UV light |
|  | **Condensation of 1,2-aminothiol and cyanobenzothiazole[8,9]**<br>• Rate constant: ~ 10 $M^{-1}s^{-1}$<br>• 1,2-aminothiol may react with cell metabolites such as pyruvate |
|  | **Inverse electron demand Diels-Alder cycloaddition with tetrazine[10-12]**<br>• Strained alkenes and alkynes may be used<br>• Rate constant: 10-$10^4$ $M^{-1}s^{-1}$<br>• Challenging to prepare both coupling reagents |
|  | **Condensation of ketone/aldehyde with activated amine[13-15]**<br>• Alkoxyamine, hydrazine or hydrazide may be used<br>• Rate constant: ~ $10^{-4}$-$10^{-1}$ $M^{-1}s^{-1}$; Optimal at *p*H 4.5-6<br>• Aniline may (or sometimes may not) catalyze the reaction<br>• Reversible feature leads to incomplete conversion and hydrolysis after removing the coupling partner. |
|  | **Modified Pictet-Spengler Ligation[13,16]**<br>• Use either aminooxy or alkylhydrazide<br>• Rate constant: ~ 1-5 $M^{-1}s^{-1}$, at *p*H 5-6<br>• Protein labeling: low conversion at 2 h; excellent conversion achieved at 12 h |
|  | **Condensation of aryl diamine and alkyl aldehyde (this work)**<br>• Rate constant: ~ 2 $M^{-1}s^{-1}$, at *p*H 7.4<br>• Use Cu(II) or Zn (II) at 2.5 μM<br>• Protein labeling: excellent conversion at 2 h |

**Figure S1.** Additional direct infusion MS characterization of the labeling of T4 lysozyme (**a**: Control experiment T4L-N68C (50 μM) directly mixed with the aldehyde Coumarin 343 dye (200 μM) in the presence of $CuSO_4$ (2.5 μM) for 2 h at RT; **b**: T4L-N68C reacted the bifunctional linker, and next, the aldehyde Coumarin 343 dye in the presence of 2.5 μM $ZnCl_2$ for 2 h at 37°C)



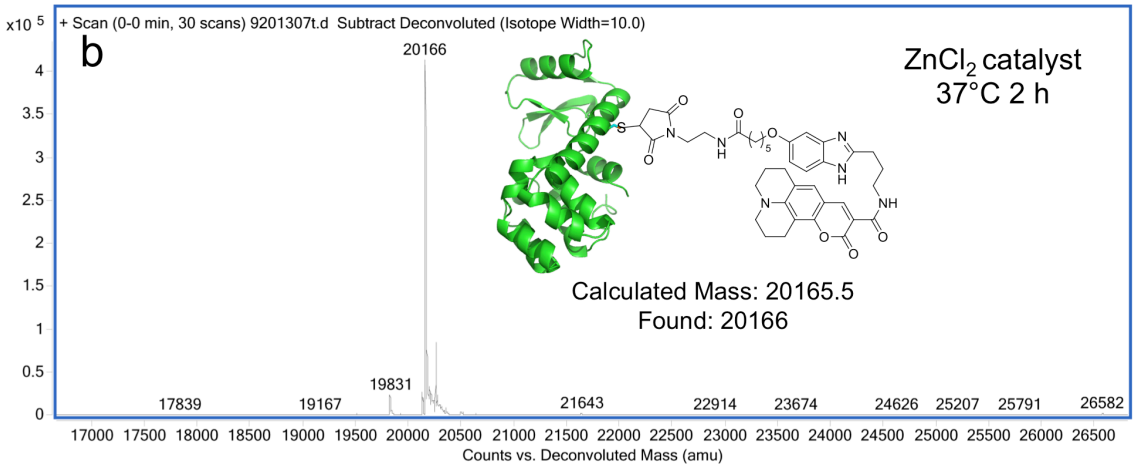
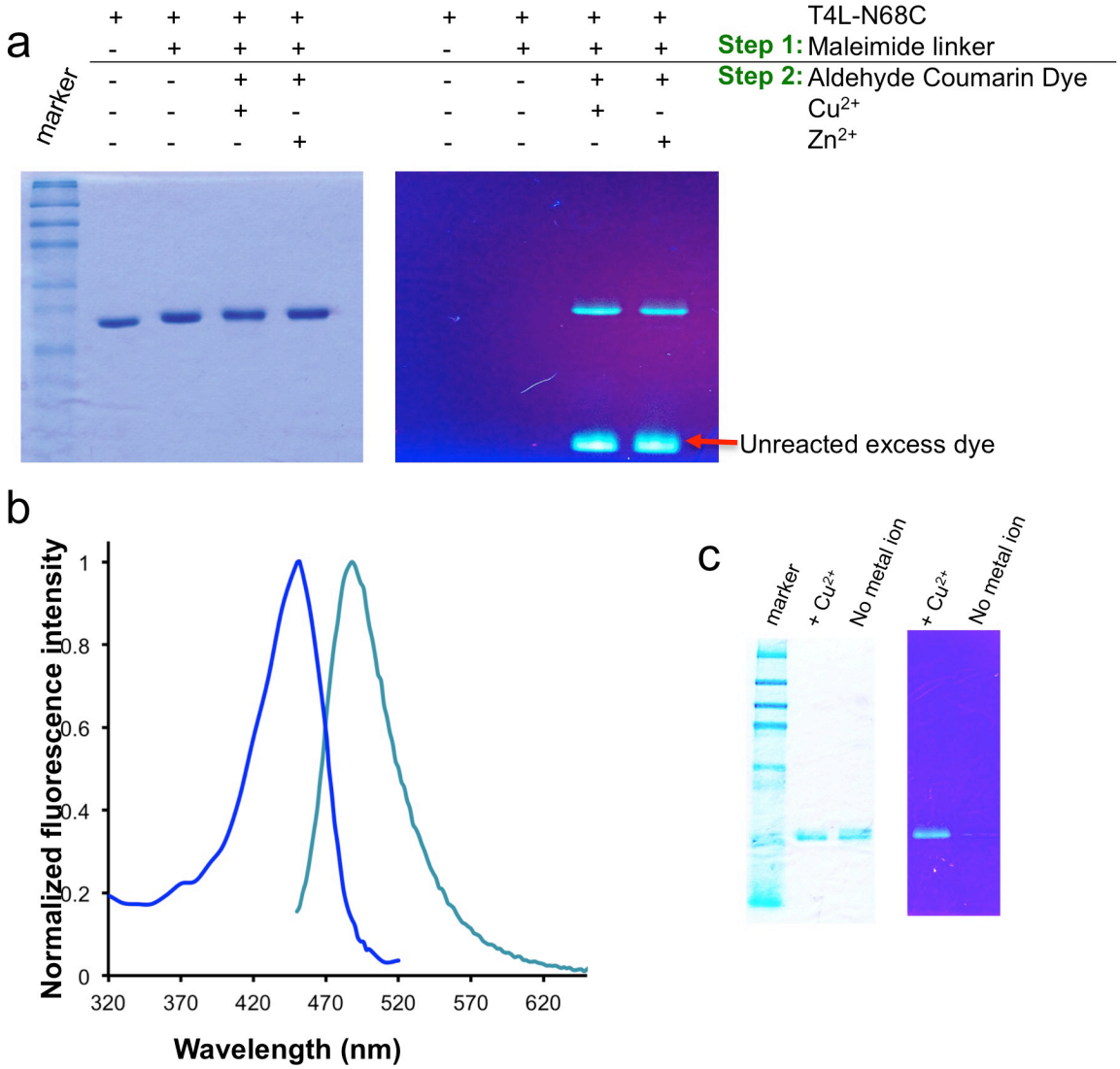



**Figure S2.** (**a**) SDS-PAGE analysis (left: Coomassie stain; right: fluorescence imaging) of T4 lysozyme proteins subjected to the two steps of sequential labeling. (**b**) Fluorescence excitation (blue) and emission (cyan) spectra of T4 lysozyme labeled with Coumarin 343. (**c**) SDS-PAGE analysis (left: Coomassie stain; right: fluorescence imaging) of T4 lysozyme proteins subjected to the two steps of sequential labeling in the presence and absence of $Cu^{2+}$. To distinguish the stable benzimidazole linkage from unstable conjugates, proteins were subjected to dialysis with phenylenediamine (20 mM) for 1 hour before SDS-PAGE analysis.



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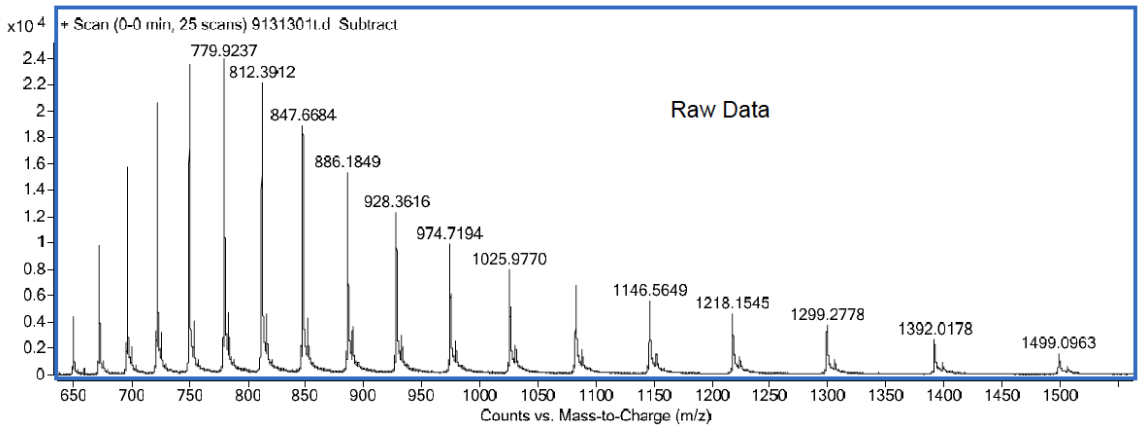
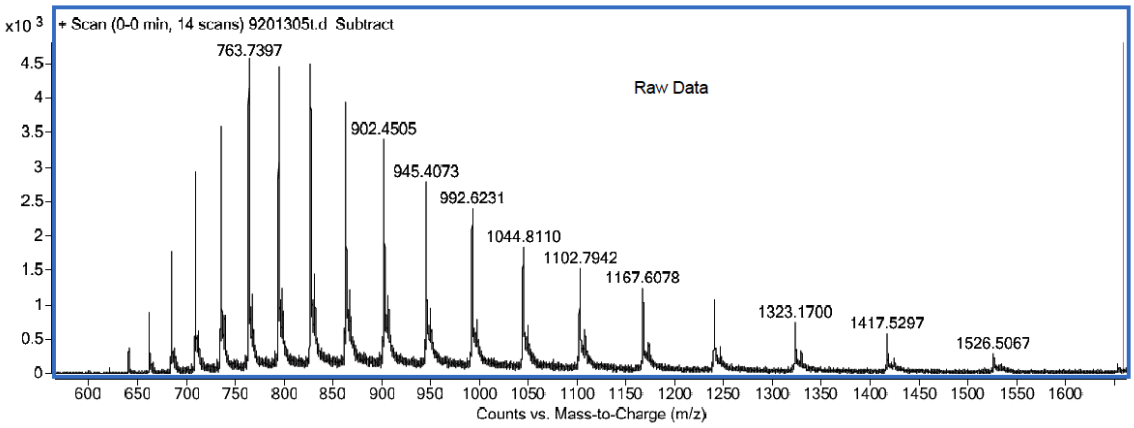
**Raw data:**

ESI MS Figure 2a before deconvolution




ESI MS Figure 2b before deconvolution

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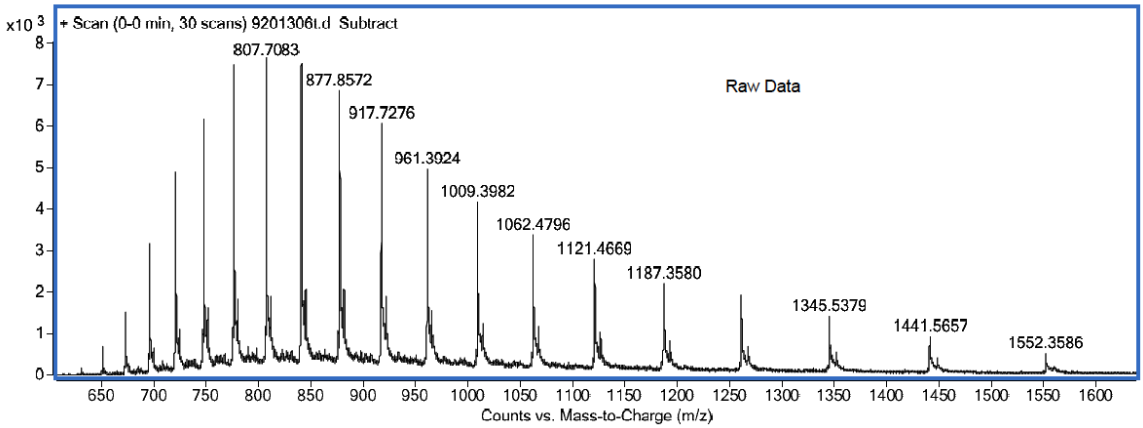

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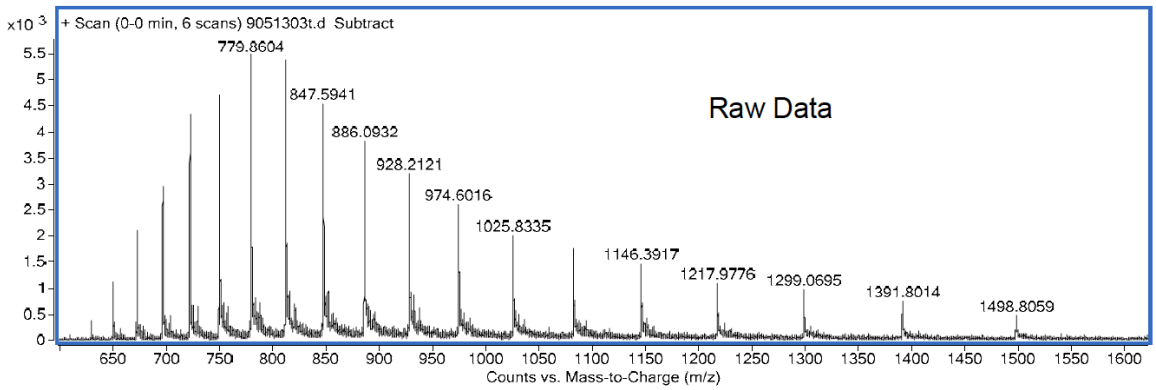


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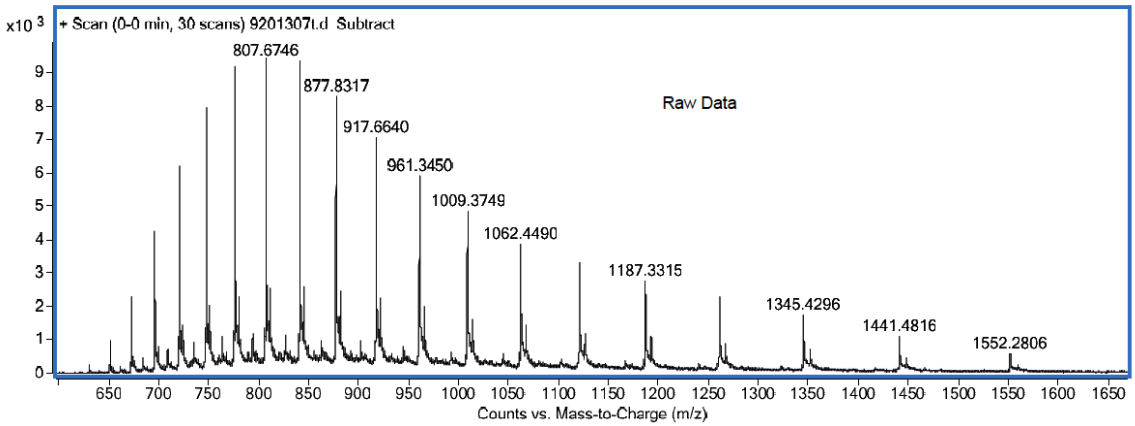
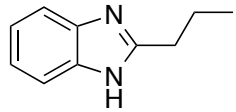
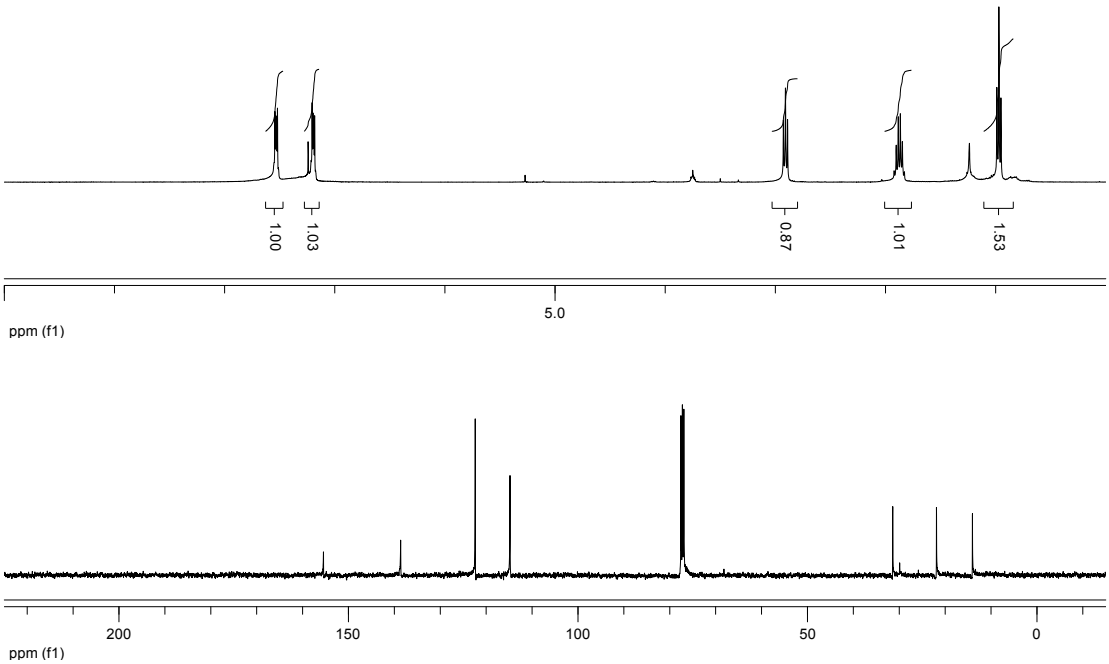


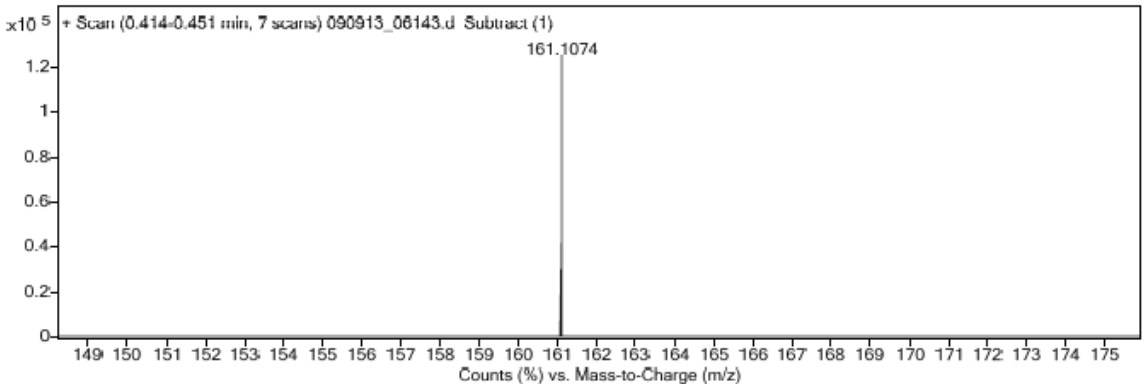
**2-propyl-1*H*-benzo[*d*]imidazole**



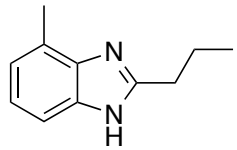


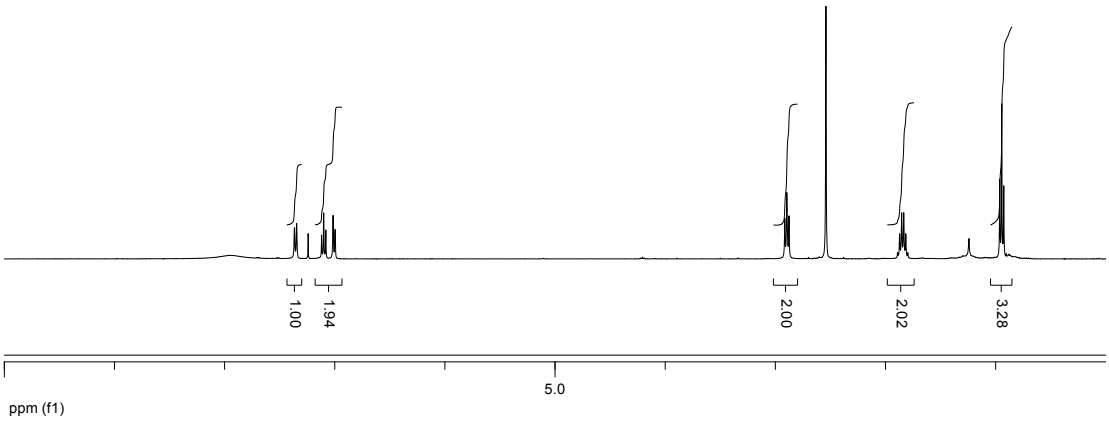


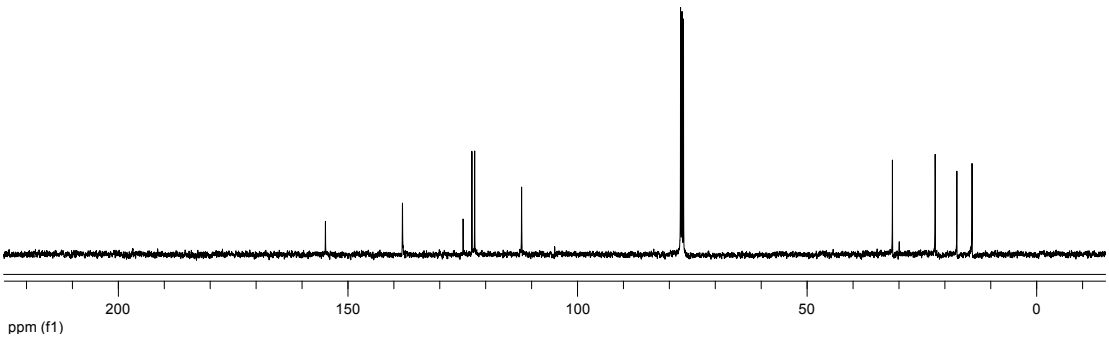


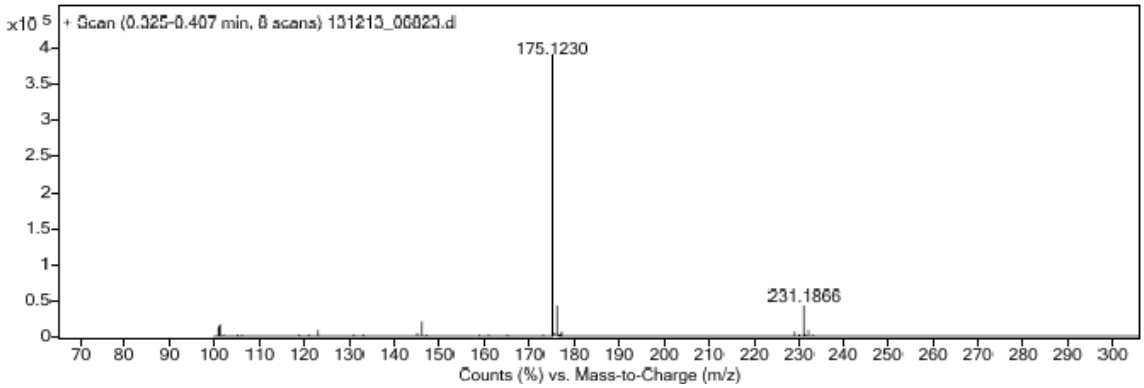


**4-methyl-2-propyl-1*H*-benzo[*d*]imidazole**

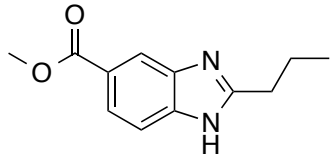


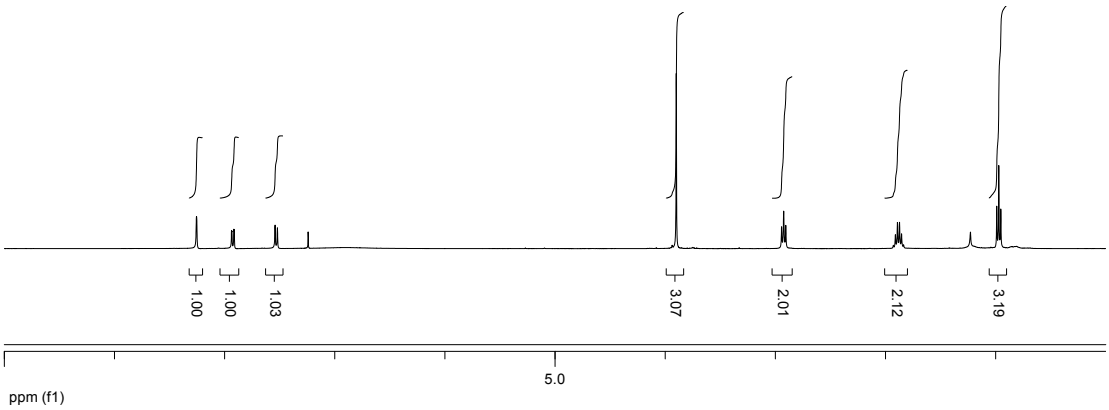



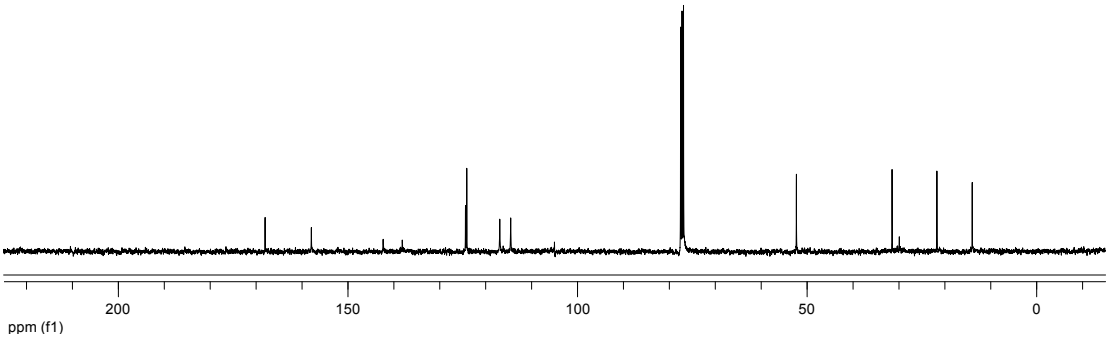



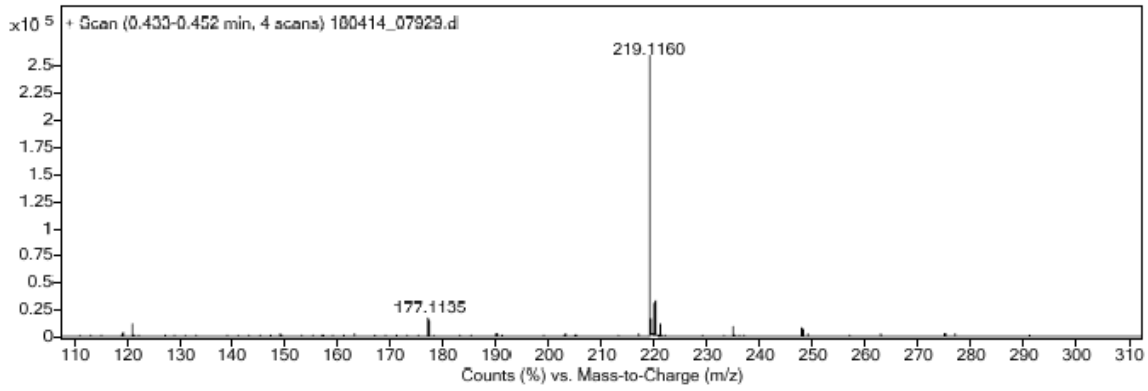



**methyl 2-propyl-1*H*-benzo[*d*]imidazole-6-carboxylate**

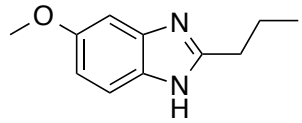


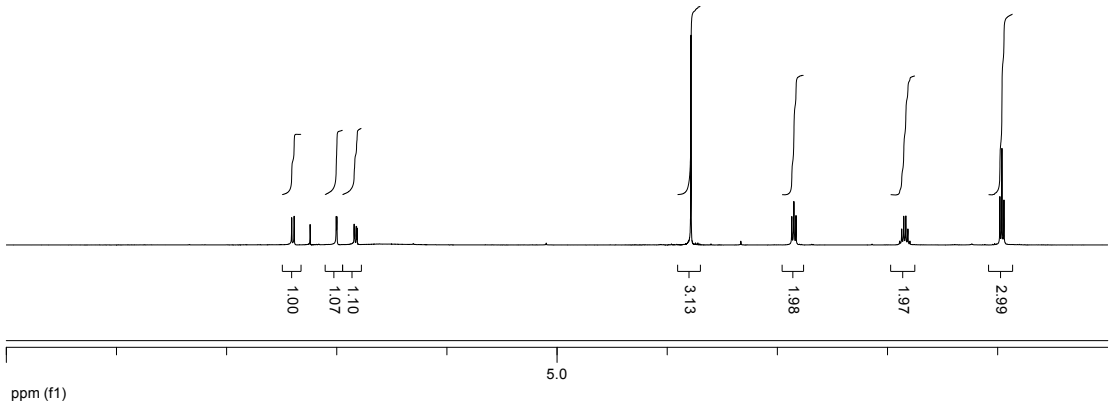



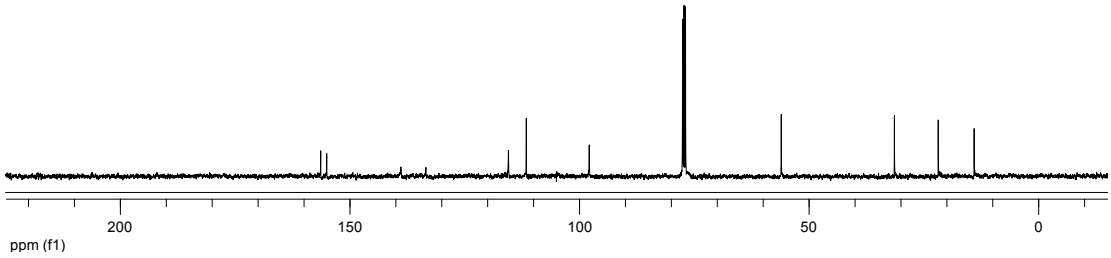



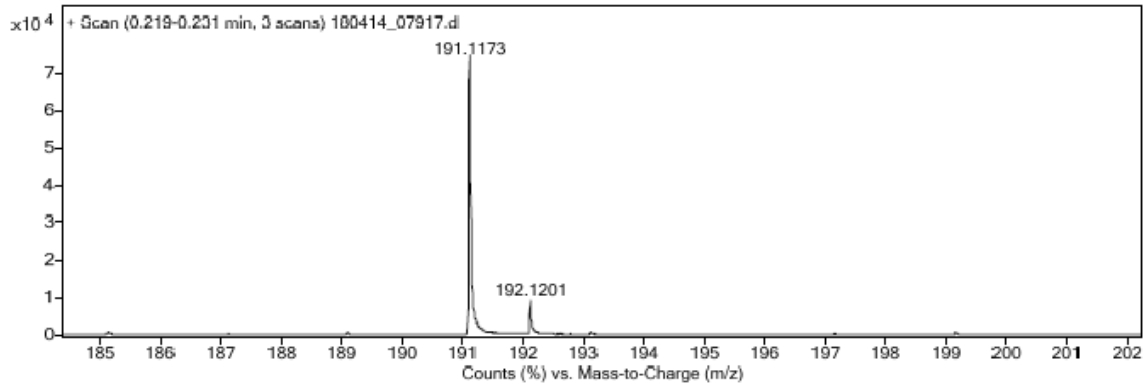



**5-methoxy-2-propyl-1*H*-benzo[*d*]imidazole**

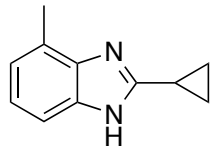


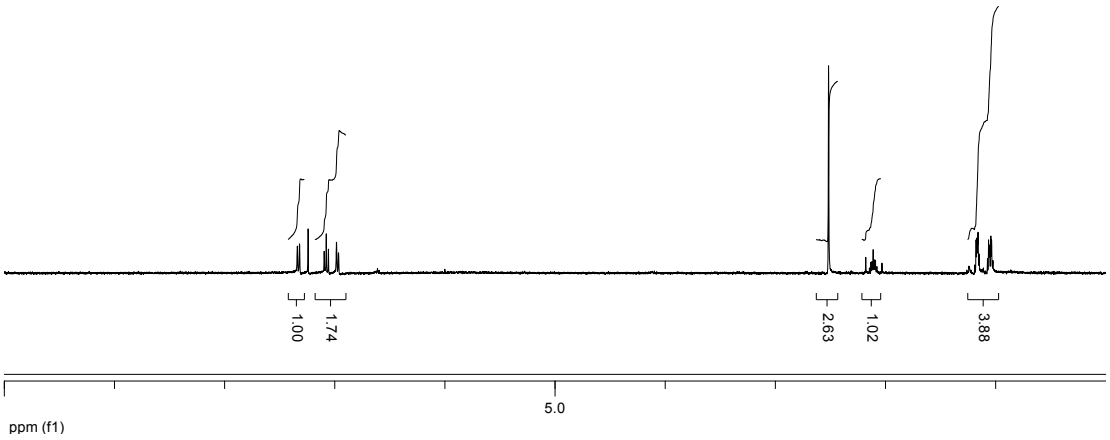



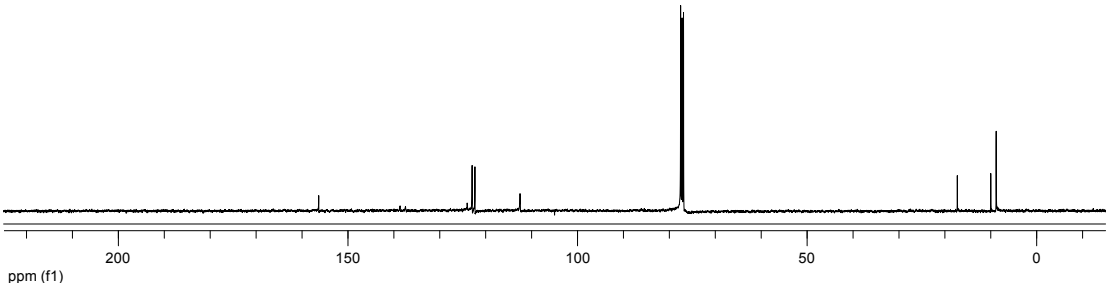



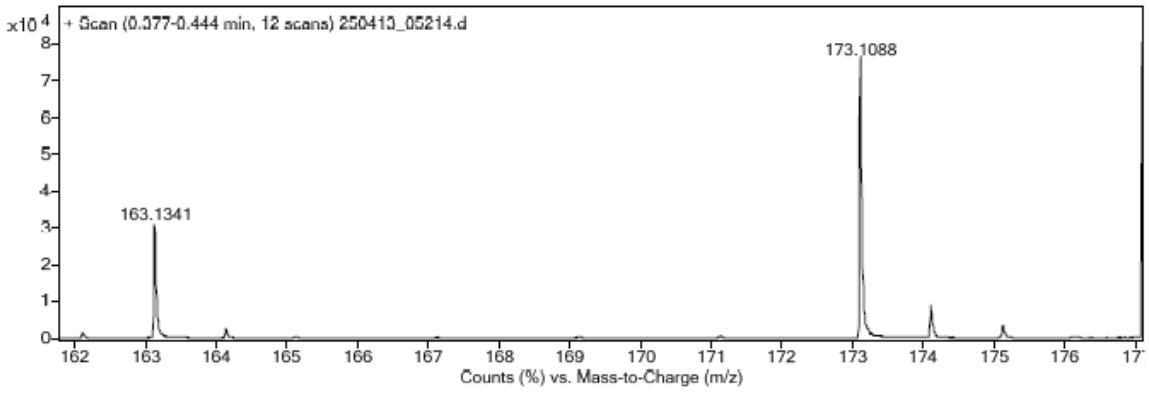



## 2-cyclopropyl-4-methyl-1*H*-benzo[*d*]imidazole



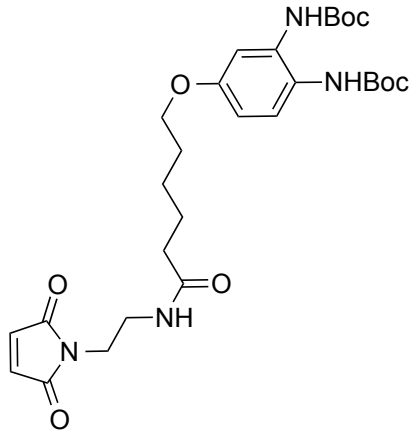



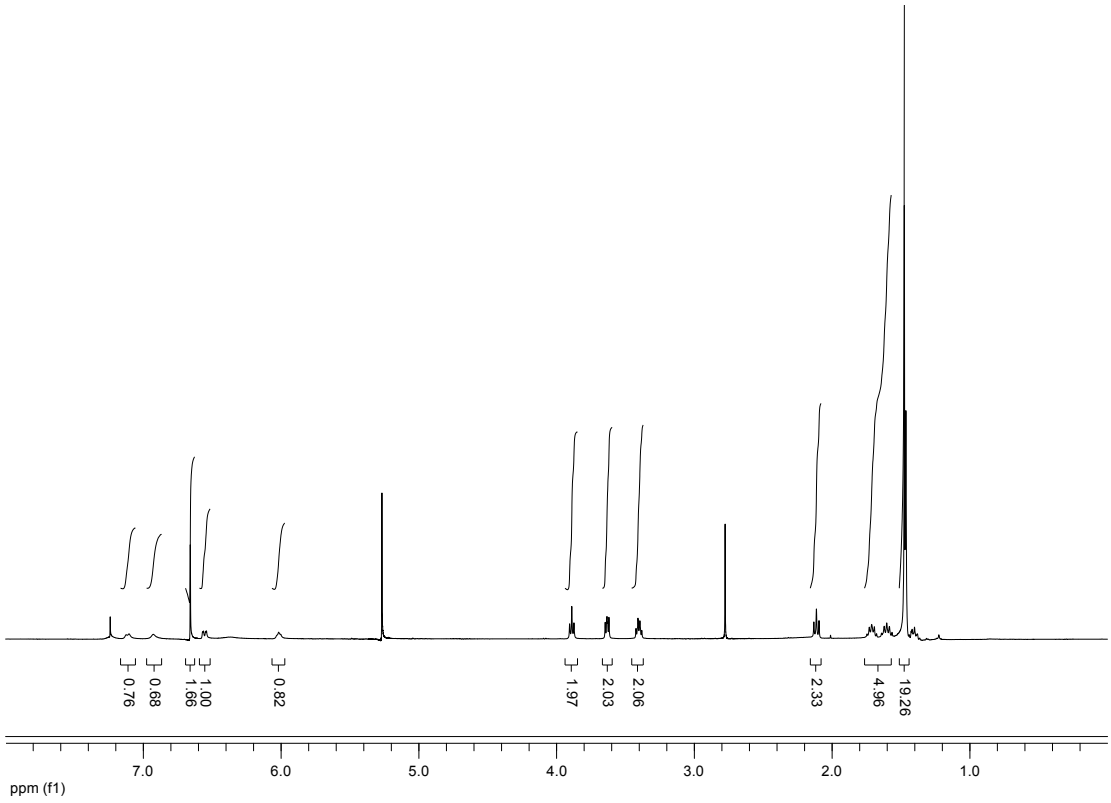



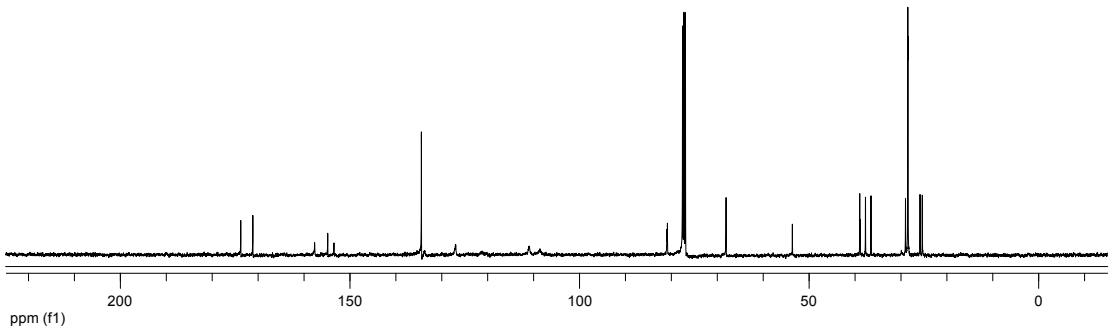



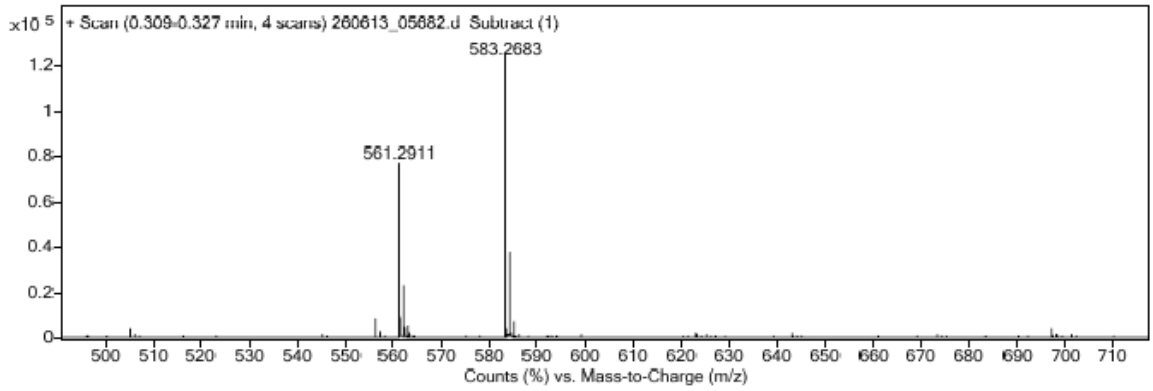
**aryl diamine maleimide linker**

**di-*tert*-butyl (4-((6-((2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)amino)-6-oxohexyl)oxy)-1,2-phenylene)dicarbamate**

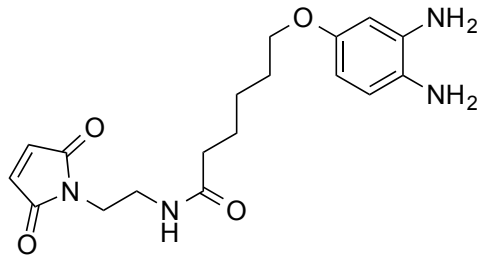


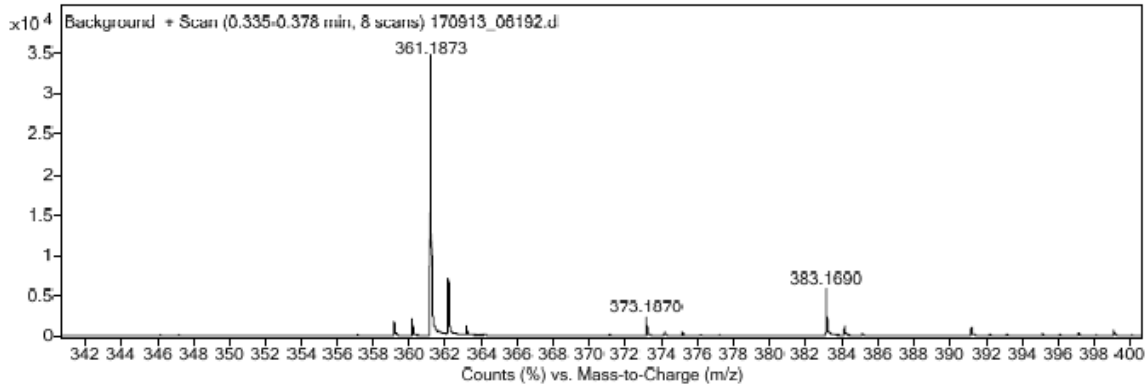






6-(3,4-diaminophenoxy)-*N*-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)hexanamide



## Coumarin 343 aldehyde dye