Supplementary Information

A Biocompatible Inverse Electron Demand Diels-Alder Reaction of Aldehyde and Tetrazine Promoted by Proline

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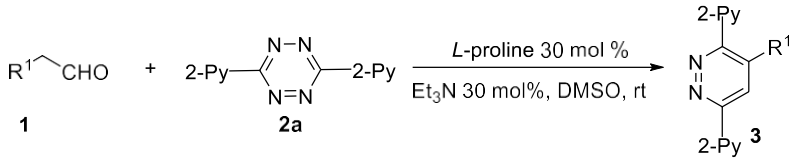
Table S1 *L*-Proline Promoted Inverse Electron Demand Diels-Alder Reaction of Aldehydes with Tetrazine **2a**[a]



Entry	Aldehyde	Time (min)	yield (%)[a]
1	Bn (**1a**)	30	93
2	PMB (**1b**)	30	88
3	PNB (**1c**)	30	97
4	*n*-Bu (**1d**)	60	87
5	Ph (**1e**)	20	99
6[b]	OBn (**1f**)	240	46

[a]Unless otherwise noted, tetrazine **2a** (0.21 mmol) and aldehyde (0.42 mmol) with *L*-proline (0.063 mmol) and Et_3N (0.063 mmol) in DMSO (1.0 mL) were stirred at rt until completion of reaction. [b]0.21 mmol of tetrazine **2a**, 2.1 mmol of aldehyde, 0.21 mmol of *L*-proline and 0.21 mmol of Et_3N were used.

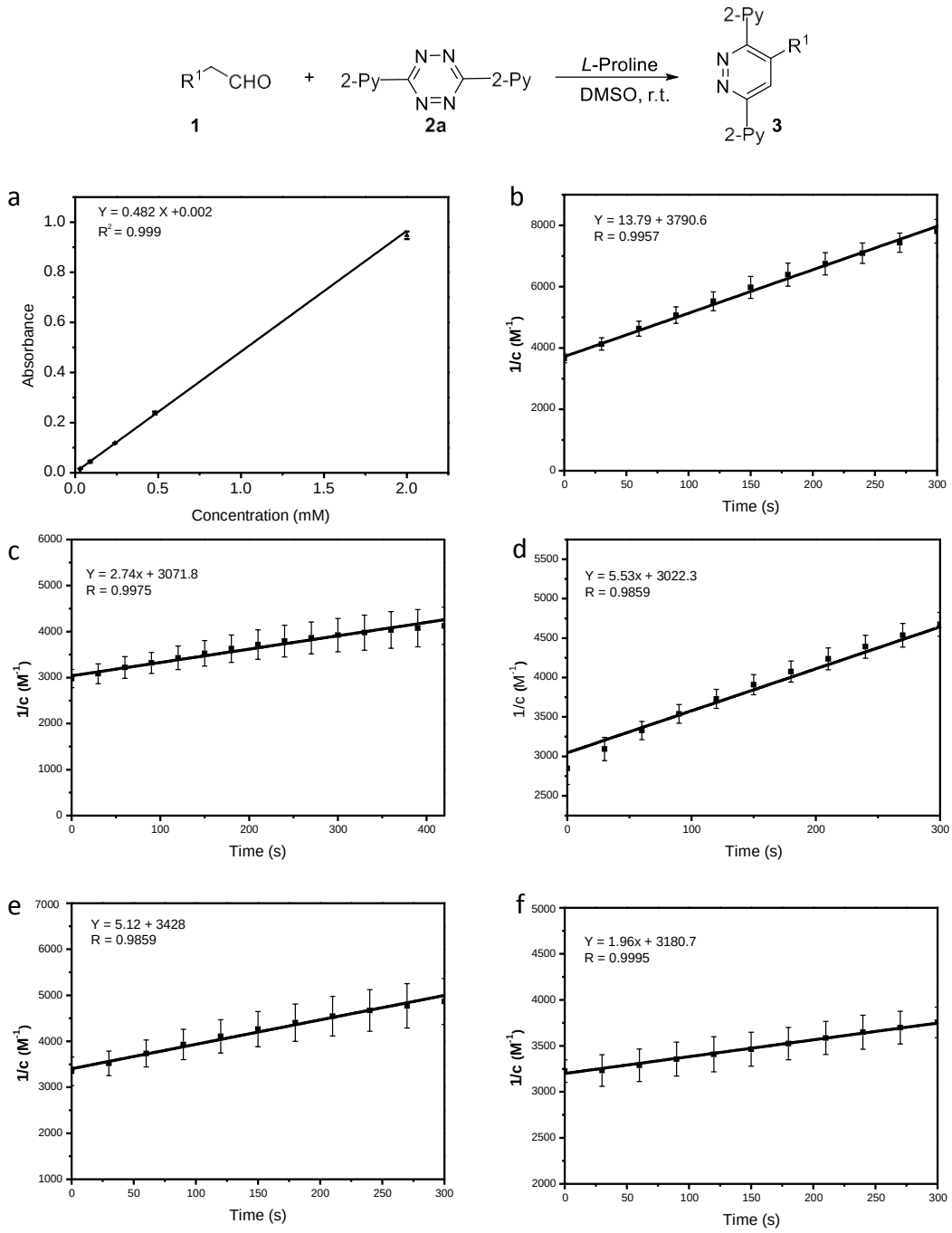


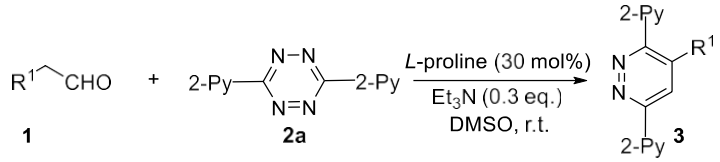
Fig. S1 Determination of rate constants for the proline-mediated inverse electron demand Diels-Alder reaction of tetrazine **2a** and aldehydes. a. Standard curve for absorbance at 534 nm versus concentration of tetrazine **2a**. Linear regression analysis of $1/C_{aldehyde}$ *vs* reaction time: aldehyde **1a** (b); aldehyde **1b** (c); aldehyde **1c** (d); aldehyde **1d** (e); aldehyde **1e** (f). Data were measured in DMSO at room temperature with $C_{\mathbf{2a}} = C_{aldehyde} = 0.3$ mM, $C_{proline} = 15$ mM. Data are the averages of three replicate experiments. Error bars are ±s.d.

General information

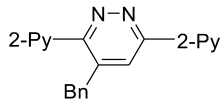
Unless otherwise noted, all reagents were obtained commercially and used without further purification. Analytical thin layer chromatography was performed on 0.20 mm Qingdao Haiyang silica gel plates. Silica gel (200-300 mesh) (from Qingdao Haiyang Chem. Company, Ltd.) was used for flash chromatography. Standard reagents and solvents were purified according to known procedures. ^{1}H, ^{13}C NMR spectra were taken on Bruker 400 or 500 MHz (^{1}H NMR), 100 or 125 MHz (^{13}C NMR) magnetic resonance spectrometer. Chemical shifts were reported in ppm from the solvent resonance as the internal standard (*d*$_6$–DMSO: δH = 2.50 ppm, δC = 39.52 ppm; $CDCl_3$, δH = 7.26 ppm, δC = 77.00 ppm). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane (δ = 0.00 ppm). Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). Coupling constants are reported as a *J* value in Hz. The gel fluorescence images were obtained on a ChampGel 5000 plus gel imager.

Compounds **1b**[1], **1c**[1], **2b**[2] and **1g**[3] were synthesized as previously reported.

General procedure for the synthesis of compounds 3

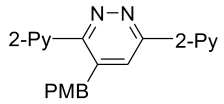


A mixture of tetrazine **2a** (0.21 mmol), aldehyde **1** (0.42 mmol), Et_3N (0.063 mmol) and *L*-proline (0.063 mmol) in DMSO (1.0 mL) was stirred at room temperature. And the resulting mixture was stirred at room temperature until disappearance of **2a** (monitored by TLC). The reaction was then diluted with ethyl acetate and washed with H_2O. Purification by column chromatography on silica gel afforded desired product **3**.



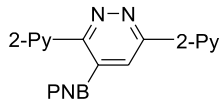
4-benzyl-3,6-di(pyridin-2-yl)pyridazine (3a)

The title compound was prepared according to the general procedure and purified by column chromatography on silica gel (petroleum ether/ethyl acetate = 2:1) to afford 63.7 mg (93%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 – 8.63 (m, 3H), 8.45 (s, 1H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.93 – 7.79 (m, 2H), 7.43 – 7.33 (m, 2H), 7.23 – 7.11 (m, 3H), 7.05 (d, *J* = 7.1 Hz, 2H), 4.54 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.0, 157.5, 156.2, 153.5, 149.4, 148.5, 141.3, 138.5, 137.1, 137.0, 129.1, 128.5, 126.5, 126.2, 125.0, 124.7, 123.7, 121.8, 38.0. FT-MS (ESI): *m/z* 325.1 $[M+1]^+$; HRMS (ESI) m/z calcd for $C_{21}H_{17}N_4$ $(M+1)^+$ 325.1453, found 325.1445.



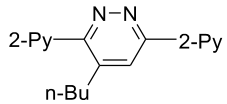
4-(4-methoxybenzyl)-3,6-di(pyridin-2-yl)pyridazine (3b)

The title compound was prepared according to the general procedure and purified by column chromatography on silica gel (eluting with petroleum ether/ethyl acetate = 2:1) to give the desired product **3b** as white solid (39.7 mg, 88% yield).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 – 8.66 (m, 3H), 8.44 (s, 1H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.94 – 7.83 (m, 2H), 7.39 (dd, *J* = 7.2, 5.1 Hz, 2H), 6.97 (d, *J* = 8.5 Hz, 2H), 6.74 (d, *J* = 8.6 Hz, 2H), 4.47 (s, 2H), 3.74 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.0, 158.1, 157.5, 156.2, 153.5, 149.4, 148.5, 141.8, 137.1, 137.0, 130.5, 130.2, 126.1, 124.9, 124.6, 123.6, 121.8, 113.9, 55.2, 37.2. FT-MS (ESI): *m/z* 355.2 $[M+1]^+$; HRMS (ESI) m/z calcd for $C_{22}H_{19}N_4O$ $(M+1)^+$ 355.1559, found 355.1550.



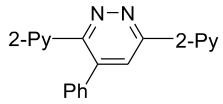
4-(4-nitrobenzyl)-3,6-di(pyridin-2-yl)pyridazine (3c)

The title compound was prepared according to the procedure and purified by column chromatography on silica gel (eluting with petroleum ether/ethyl acetate = 1:1) to give the desired product **3c** as white solid (45.7 mg, 97% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (d, *J* = 7.9 Hz, 1H), 8.74 – 8.65 (m, 2H), 8.46 (s, 1H), 8.15 (d, *J* = 7.8 Hz, 1H), 8.13 – 8.03 (m, 2H), 7.98 – 7.82 (m, 2H), 7.47 – 7.35 (m, 2H), 7.28 (d, *J* = 9.2 Hz, 2H), 4.70 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.5, 157.6, 155.7, 153.0, 149.4, 148.5, 146.6, 146.5, 139.4, 137.4, 137.2, 126.6, 125.0, 124.9, 124.0, 123.7, 121.9, 38.3. FT-MS (ESI): *m/z* 370.1 $[M+1]^+$; HRMS (ESI) m/z calcd for $C_{21}H_{16}N_5O$ $(M+1)^+$ 370.1304, found 370.1296.



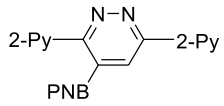
4-butyl-3,6-di(pyridin-2-yl)pyridazine (3d)

The title compound was prepared according to the general procedure and purified by column chromatography on silica gel (petroleum ether/ethyl acetate = 4:1) to afford 53.5 mg (87%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (t, *J* = 7.3 Hz, 3H), 8.50 (s, 1H), 8.09 (d, *J* = 7.9 Hz, 1H), 7.91 (t, *J* = 7.8 Hz, 2H), 7.45 – 7.36 (m, 2H), 3.09 (t, *J* = 8.0 Hz, 2H), 1.65 – 1.55 (m, 2H), 1.38 – 1.31 (m, 2H), 0.87 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, CDCl3) δ 159.2, 157.3, 156.5, 153.7, 149.4, 148.6, 141.3, 138.5, 137.1, 136.95, 125.6, 124.8, 124.7, 123.5, 121.8, 32.0, 32.0, 22.6, 13.8. EI-MS: *m/z* 290.0 $[M]^+$; HRMS (EI) m/z calcd for $C_{18}H_{18}N_4$ $[M]^+$ 290.1531, found 290.1526.



4-phenyl-3,6-di(pyridin-2-yl)pyridazine (3e)

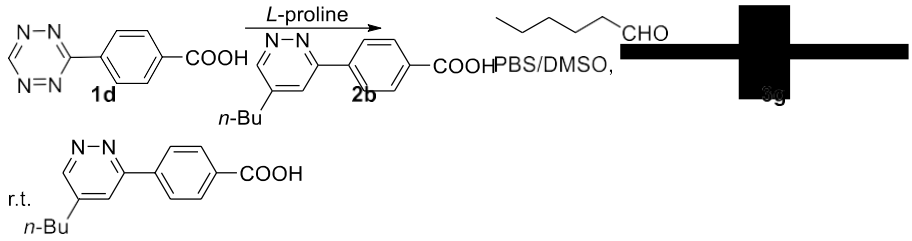
The title compound was prepared according to the procedure and purified by column chromatography on silica gel (eluting with petroleum ether/ethyl acetate = 1:1) to give the desired product **3e** as white solid (39.5 mg, 99% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (d, *J* = 8.0 Hz, 1H), 8.74 (d, *J* = 4.7 Hz, 1H), 8.67 (s, 1H), 8.49 (d, *J* = 4.7 Hz, 1H), 7.94 (dd, *J* = 7.7, 1.4 Hz, 1H), 7.90 (d, *J* = 7.7 Hz, 1H), 7.81 (td, *J* = 7.7, 1.4 Hz, 1H), 7.45 – 7.40 (m, 1H), 7.37 – 7.27 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.4, 157.7, 155.8, 153.4, 149.5, 149.1, 140.5, 137.3, 136.9, 136.6, 129.0, 128.5, 125.7, 124.9, 124.9, 123.4, 121.9. FT-MS (ESI): *m/z* 311.1 $[M+1]^+$; HRMS (ESI) m/z calcd for $C_{20}H_{15}N_4$ $(M+1)^+$ 311.1297, found 311.1289.



4-(benzyloxy)-3,6-di(pyridin-2-yl)pyridazine (3f)

The title compound was prepared according to the general procedure (with 1 equiv. of *L*-proline and 1 equiv. of Et_3N) and purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 60:1) to afford 33.1 mg (46%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (d, *J* = 4.7 Hz, 1H), 8.77 (d, *J* = 8.0 Hz, 1H), 8.74 (d, *J* = 4.4 Hz, 1H), 8.30 (s, 1H), 8.05 (d, *J* = 7.9 Hz, 1H), 7.91 (t, *J* = 7.1 Hz, 2H), 7.48 – 7.29 (m, 7H), 5.43 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.1, 156.8, 153.8, 153.3, 152.1, 149.3, 149.3, 137.2, 136.5, 135.0, 128.7, 128.3, 127.1, 125.0, 124.9, 123.7, 122.1, 106.6, 70.2. FT-MS (ESI): *m/z* 341.1 $[M+1]^+$; HRMS (ESI) m/z calcd for $C_{21}H_{17}N_4O$ $(M+1)^+$ 341.1402, found 341.1394.

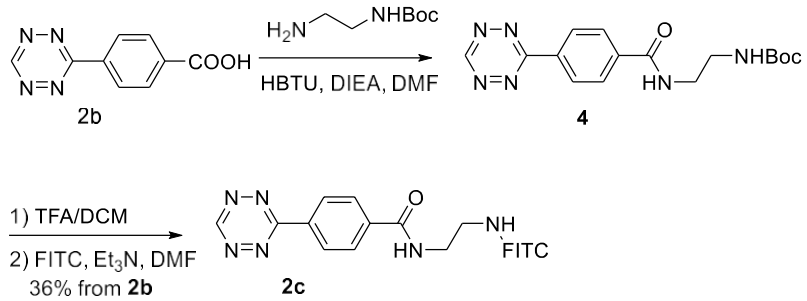
Synthesis of compound 3g



4-(5-butylpyridazin-3-yl)benzoic acid (3g)

To solution of **2b** (40.6 mg, 0.2 mmol) and *L*-proline(23.7mg, 0.2 mmol) in PBS/DMSO (3:7, 1 mL) was added **1d** (46 μL, 0.4 mmol) and the resulting mixture was stirred at rt for 2h. Ethyl acetate (30 mL) was then added and the solution was washed with water (15 mL x 3) and dried over Na_2SO_4. The title compound was obtained after purification by column chromatography on silica gel (36 mg, 70%). ^{1}H NMR (400 MHz, DMSO) δ 9.20 (s, 1H), 8.31 (d, *J* = 8.2 Hz, 2H), 8.22 (s, 1H), 8.12 (d, *J* = 8.2 Hz, 2H), 2.72 (t, *J* = 7.7 Hz, 2H), 1.77 – 1.57 (m, 2H), 1.41 – 1.29 (m, 2H), 0.93 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 166.98, 157.15, 151.74, 143.08, 140.09, 131.89, 129.91, 127.16, 124.41, 31.48, 31.41, 21.81, 13.70; HRMS (ESI) m/z calcd for $C15H_{17}N_2O_2$ $(M+1)^+$ 257.1290, found 257.1292.

Synthesis of compound 2c



To a solution of **2b** (30 mg, 0.15 mmol), DIEA (56 μL, 0.3 mmol) and *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU, 85 mg, 0.225 mmol) in DMF (1.0 mL) at room temperature was added *N*-Boc- ethylenediamine (28.8 mg, 0.18 mmol), and the resulting mixture were stirred at room temperature until disappearance of **2b** (monitored by TLC). Upon dilution with ethyl acetate, the solution was washed with water and purified by column chromatography on a short silica gel column to afford compound **4** (23.3 mg) as crude product.

The compound **4** (10.2 mg) was then treated with a solution of trifluoroacetic acid in DCM (30%) for 20 min and the solvent and volatile reagent was removed *in vacuo*. To the resulting residue was added DMF (150 μL), Et_3N (16 μL) and fluorescein isothiocyanate (FITC, 11.5 mg, 0.03 mmol) and stirred at room temperature for 3h in dark. The title compound was obtained after purification by RP-HPLC on C18 column (14.8 mg, 36% from **2b**). The purity of the compound was determined by RP-HPLC on C18 column. MS （ESI）m/z 634.35 $[M+1]^+$; HRMS (ESI) m/z calcd for $C_{32}H_{24}O_6N_7S$ $(M+1)^+$ 634.1509, found 634.1503.

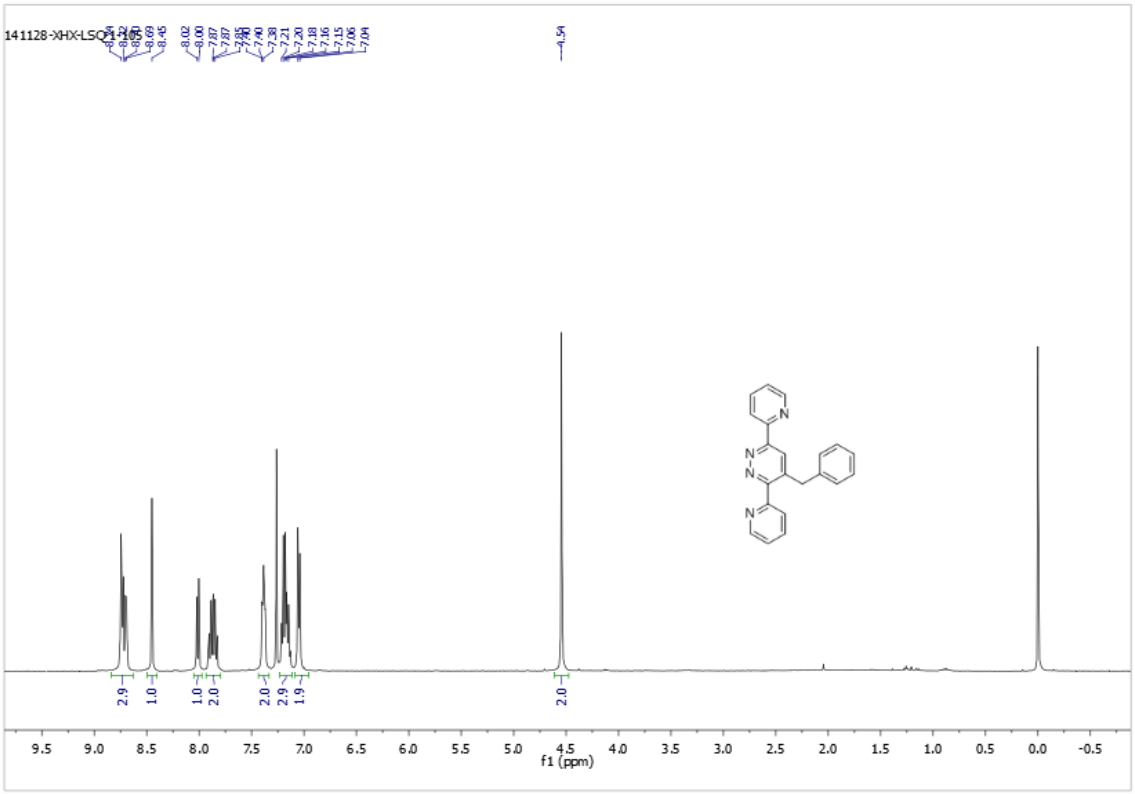
References

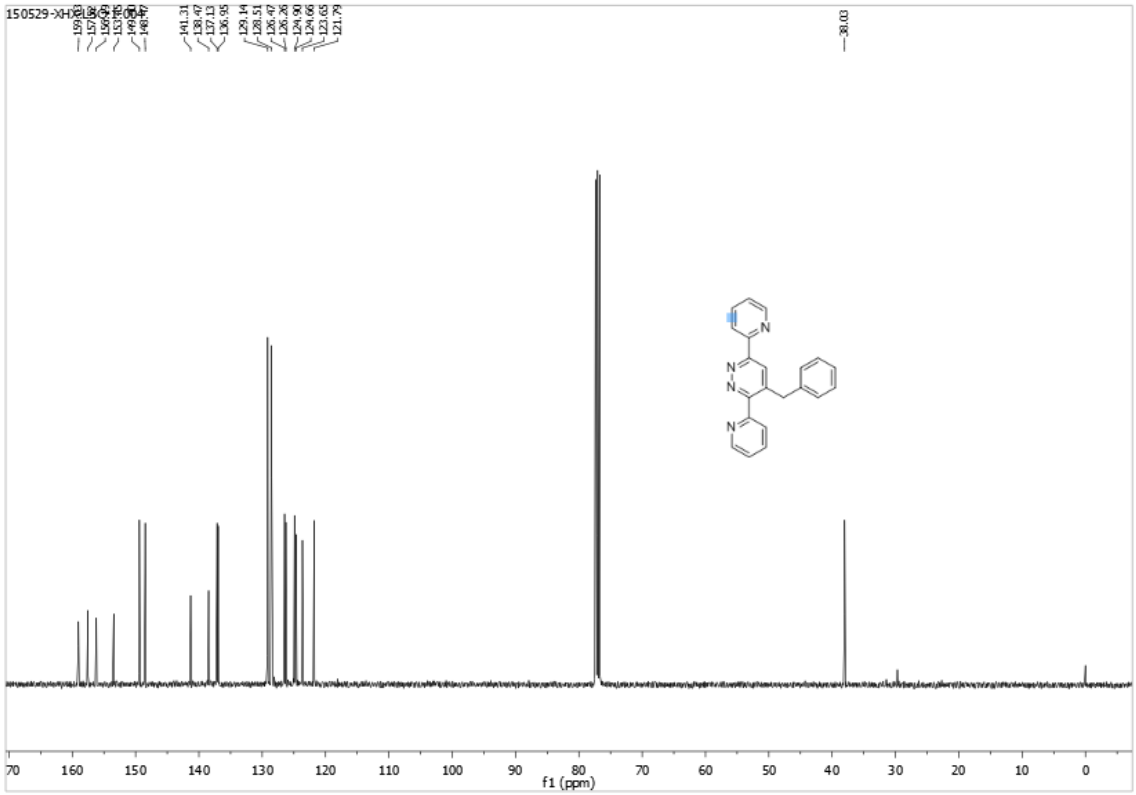
1. J. W. Yang, M. T. Hechavarria Fonseca, B. List, *Angew. Chem. Int. Ed.* 2004, **43**, 6660.

2. M. R. Karver, R. Weissleder and S. A. Hilderbrand, *Bioconjug Chem*, 2011, **22**, 2263.

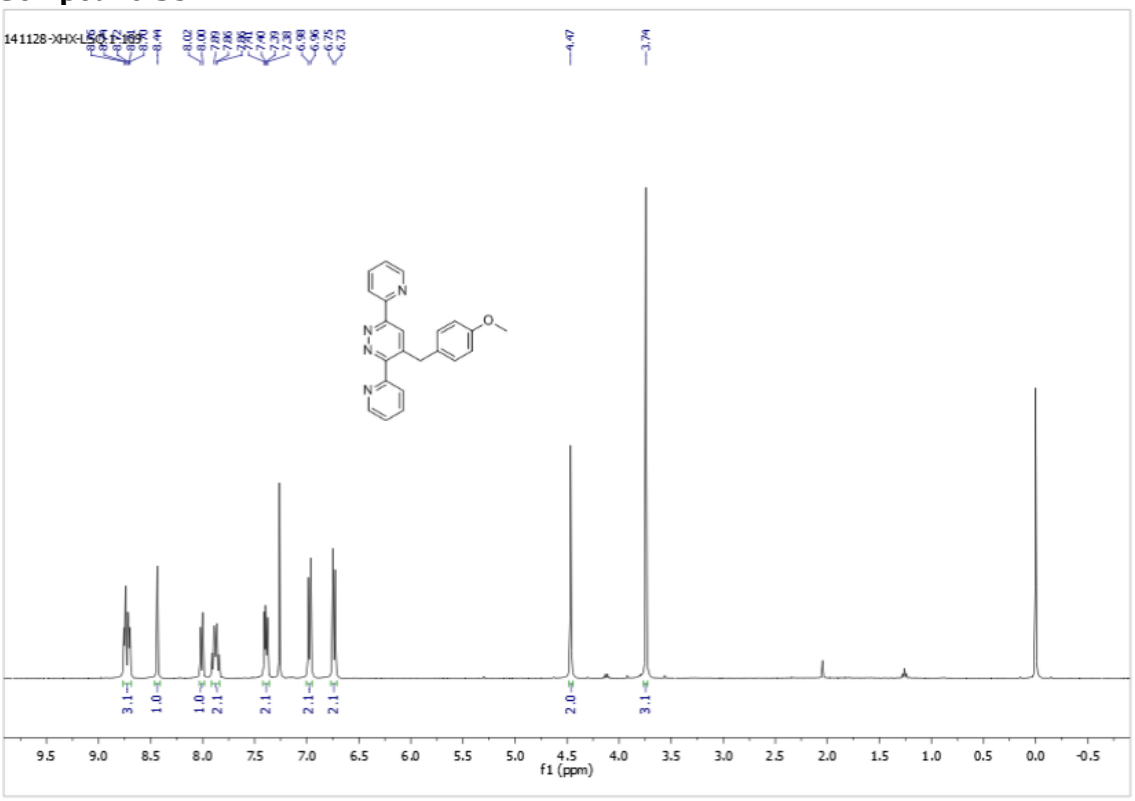
3. J. Grunewald, H. E. Klock, S. E. Cellitti, B. Bursulaya, D. McMullan, D. H. Jones, H. P. Chiu, X. Wang, P. Patterson, H. F. Zhou, J. Vance, E. Nigoghossian, H. Tong, D. Daniel, W. Mallet, W. J. Ou, T. Uno, A. Brock, S. A. Lesley and B. H. Geierstanger, *Bioconjugate Chem.*, 2015, **26**, 2554.

1H and 13C-NMR spectra
Compound 3a





Compound 3b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2.1
2.1
2.1
2.1
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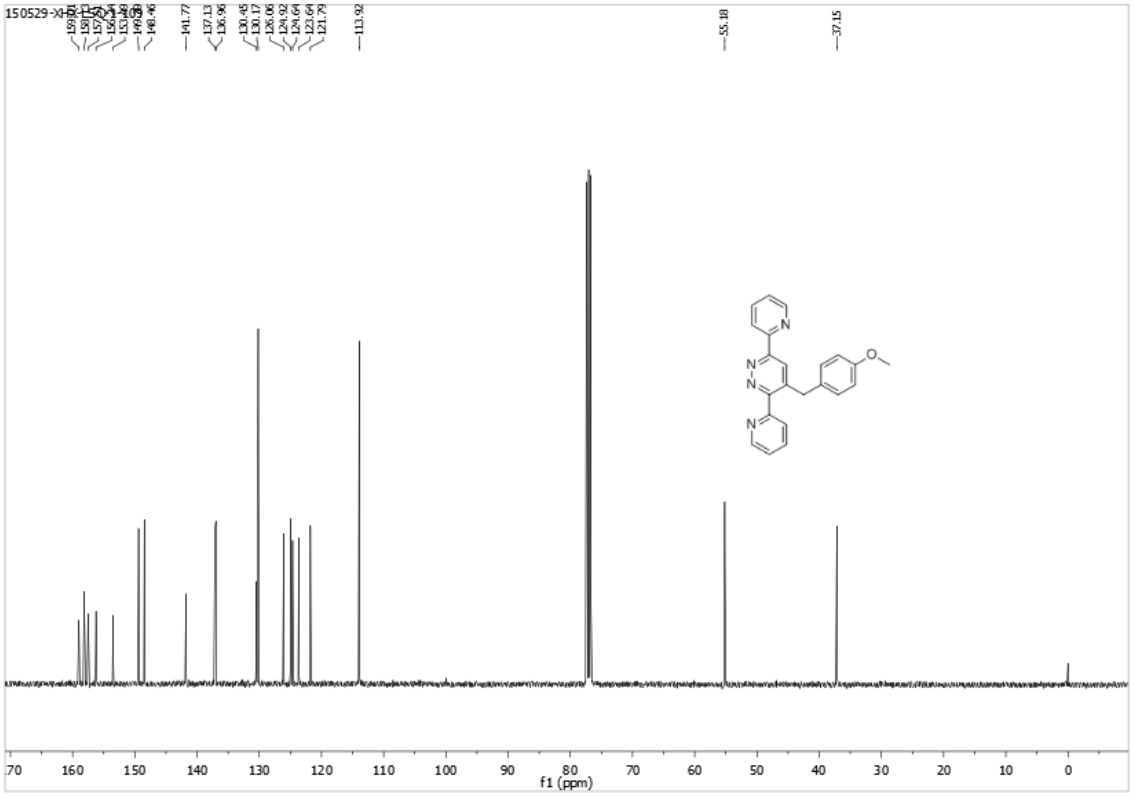
3.1
f1 (ppm)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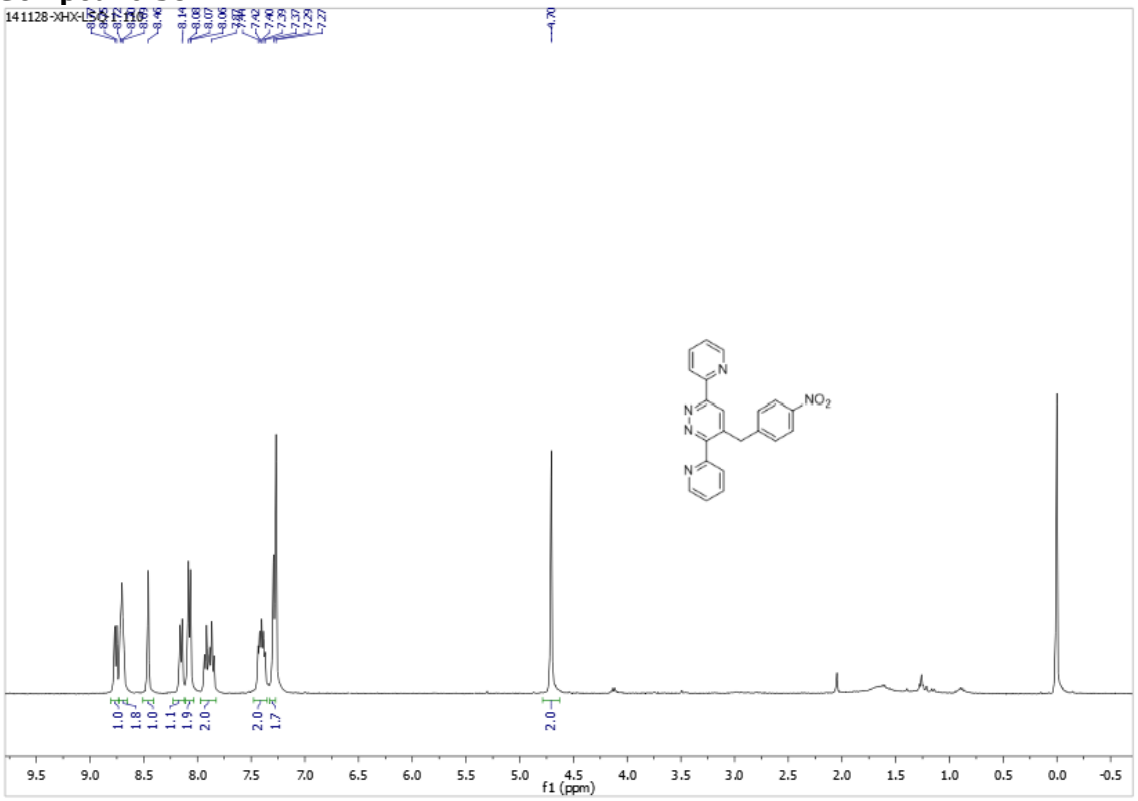
55.18
37.15
f1 (ppm)

Compound 3c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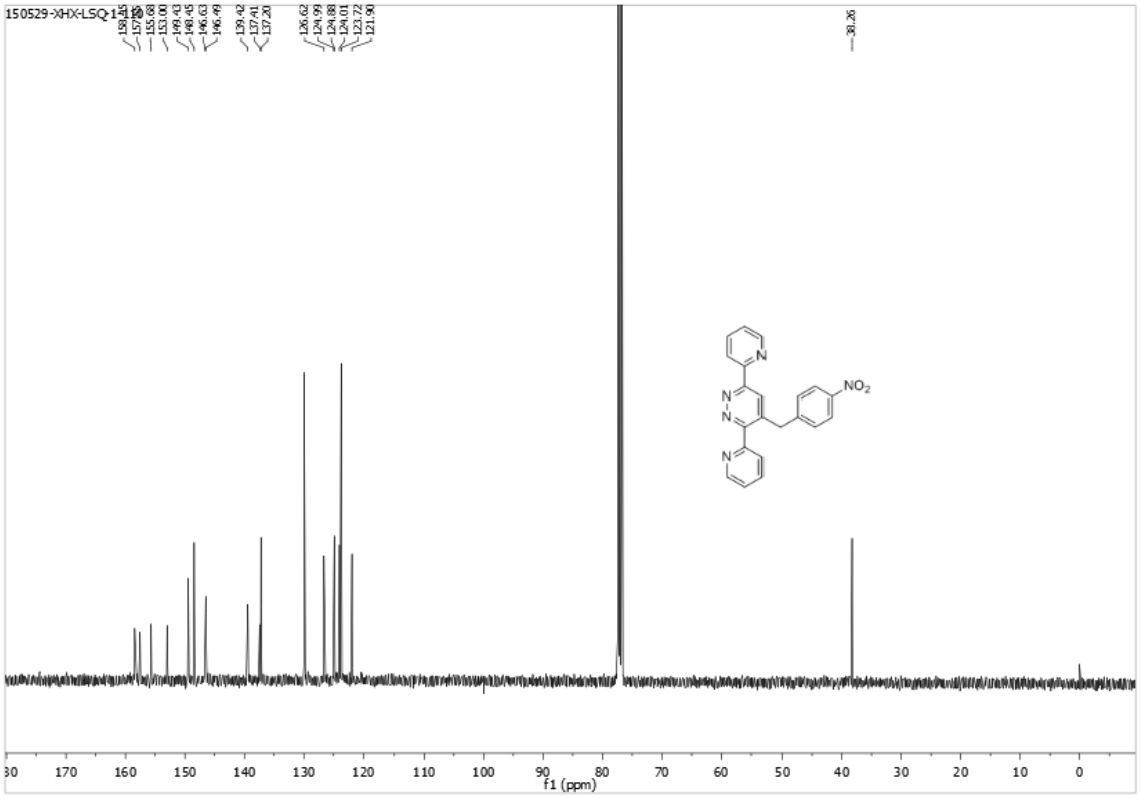
f1 (ppm)


150529-XHX-LSQ-1
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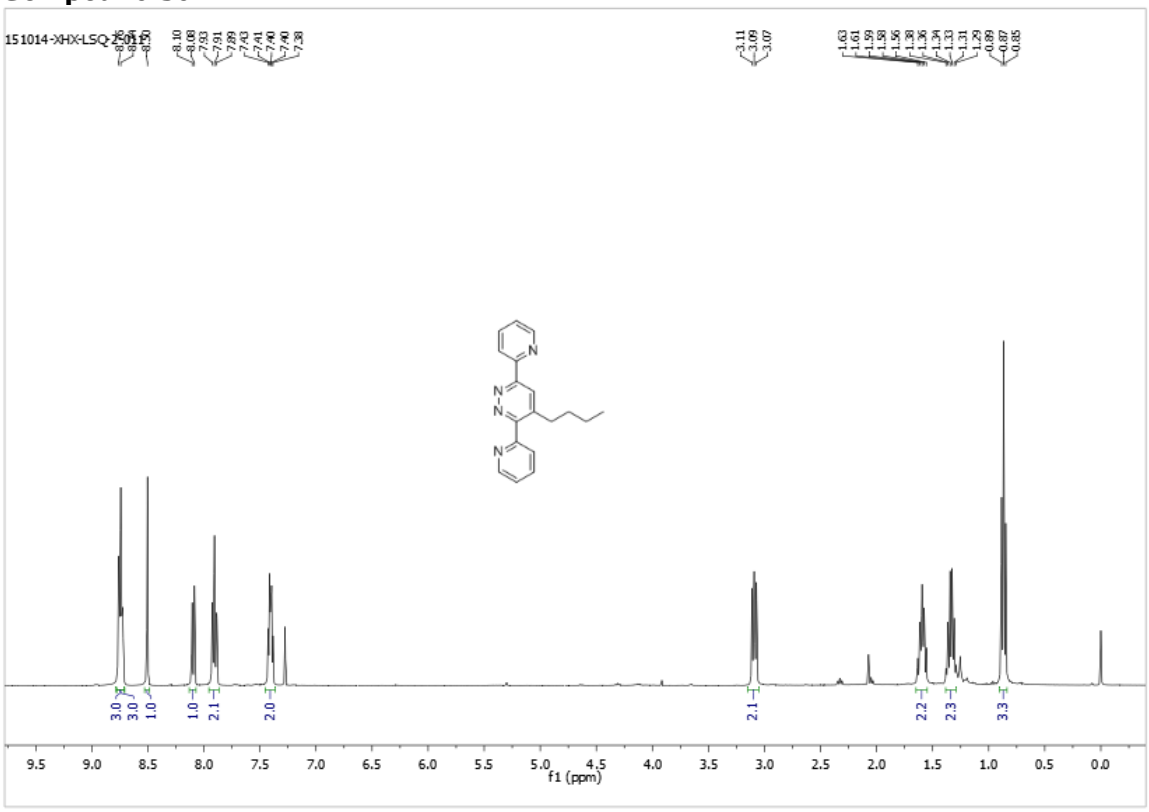
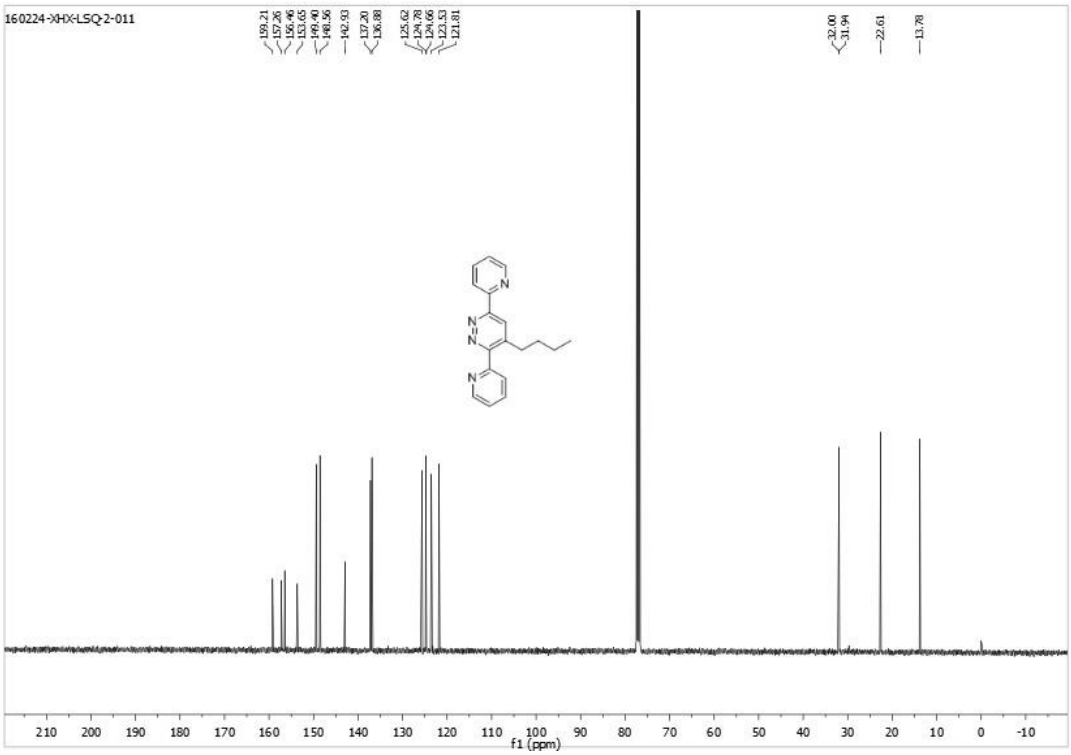
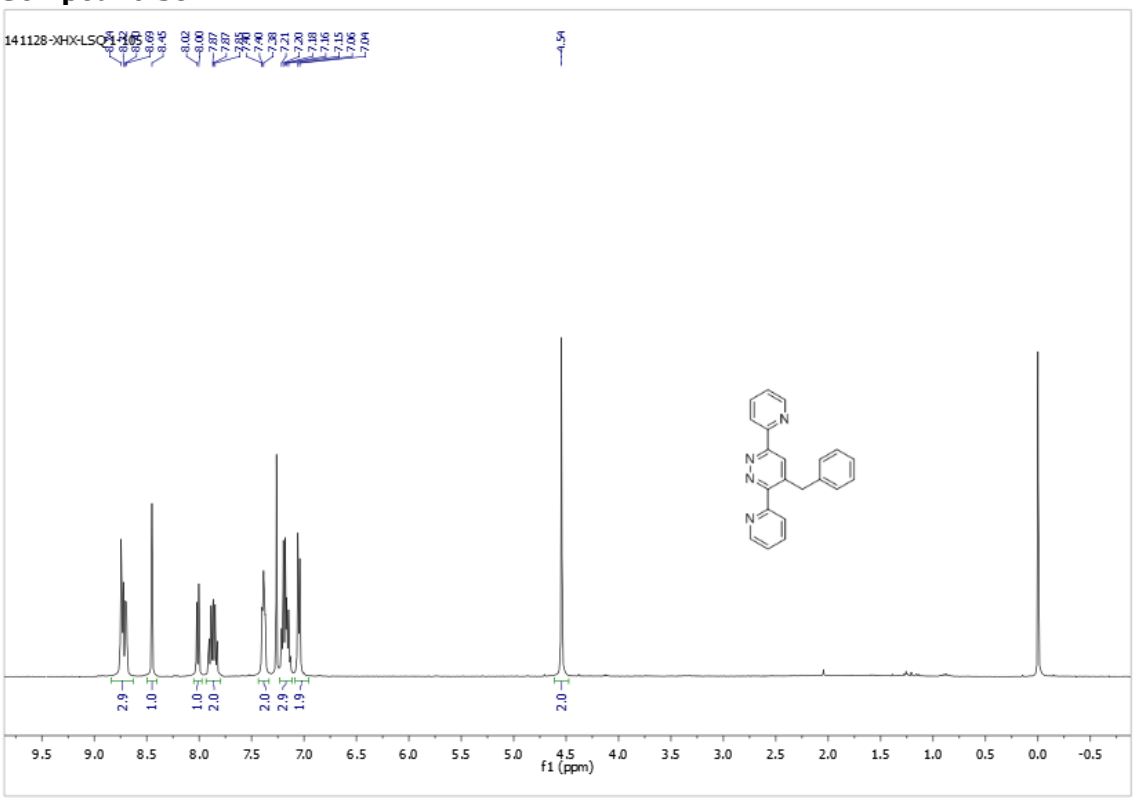
f1 (ppm)

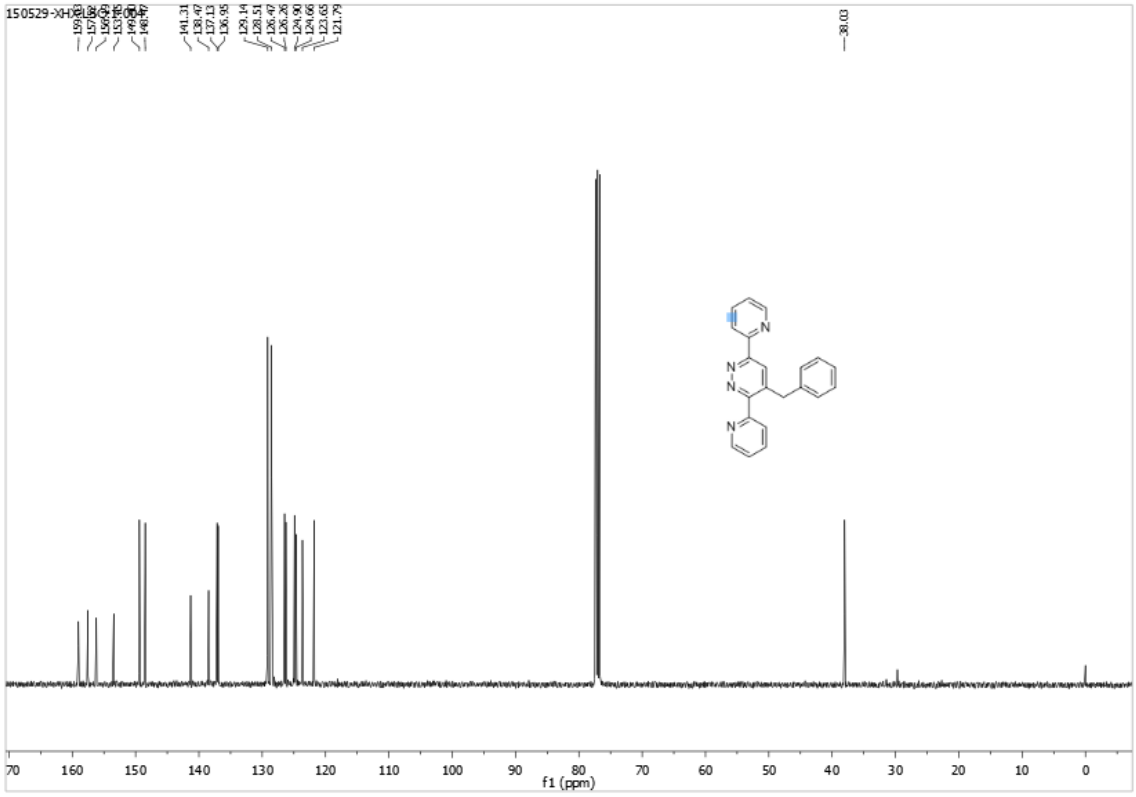
Compound 3d



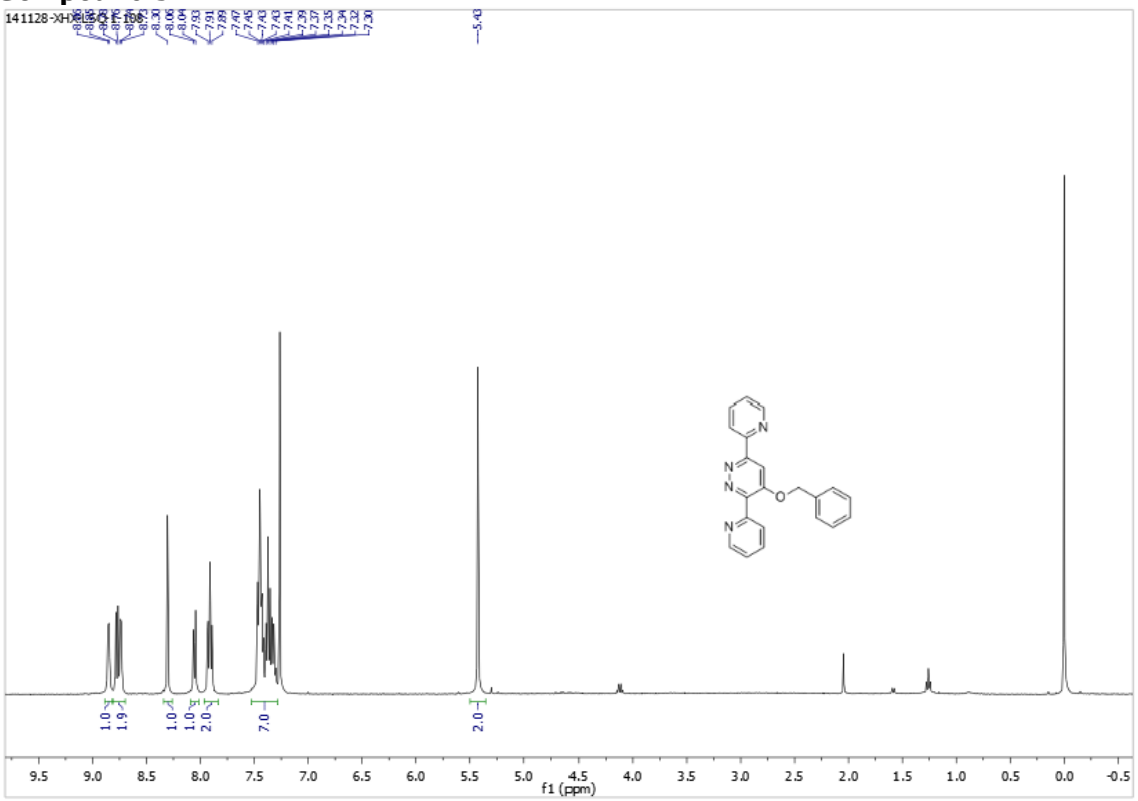


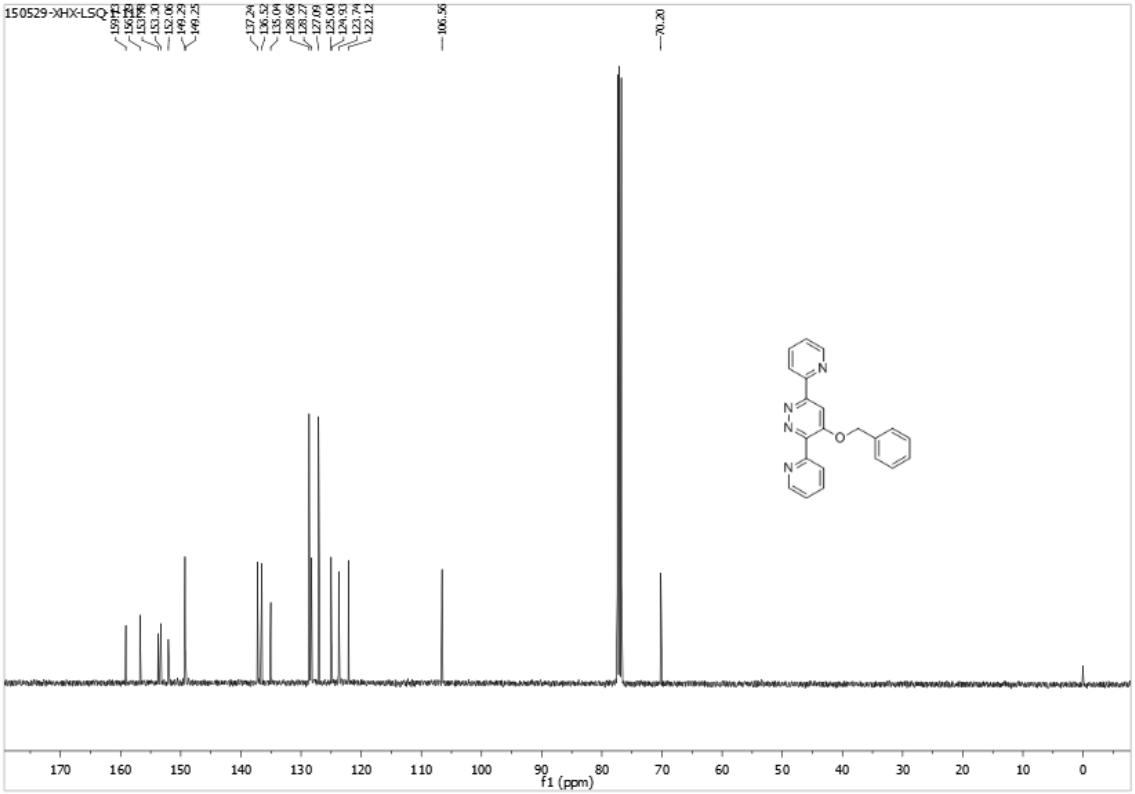
Compound 3e





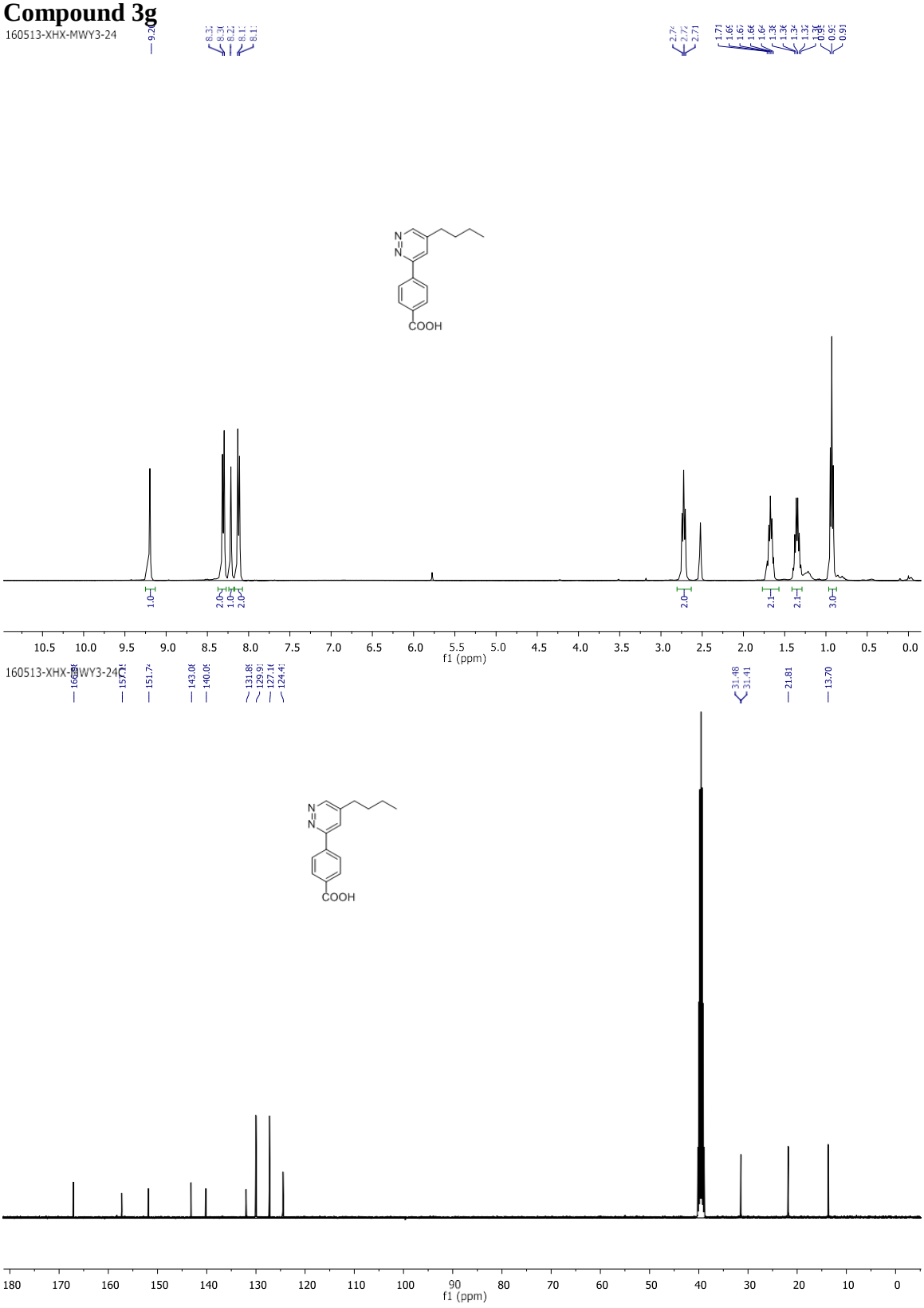
Compound 3f





Compound 3g

160513-XHX-MWY3-24



HPLC trace of compound 2c

<色谱图>