Supplemental Information

Nucleosides modification based flexizymes with versatile activity for tRNA aminoacylation

Xin-Dan Zhang, ‡[a] Yi-Shen Wang, ‡[a] Hua Xiang, ‡[a] Li-Wen Bai, [a] Peng Cheng, [a] Kai Li, [c] Rong Huang, [a] Xiao-Lei Wang [b] and Xinxiang Lei *[ab]

[a] School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China
[b] State Key Laboratory of Applied Organic Chemistry, College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China
[c] College of Life Sciences, South-Central Minzu University, Wuhan, 430074, China.
‡ These authors contributed equally to this work.

Contents

Supplementary Methods 5
Materials and Methods 5
General procedure for synthesis of DBE substrates 5
General procedure for synthesis of CME substrates 5
Characterizations of substrates 7
The synthesis of the chemically modified RNAs 12
Acylation of microhelix 12
The analysis of Acid-Urea PAGE 12
Table S1. The names, sequences, and structures of the involved RNAs 14
Supplementary Figure 1. The chemical modification methods for nucleosides 15
Supplementary Figure 2. The chemical modification methods for Fx. 16
Supplementary Figure 3. Acylation of microhelix with $^{N\text{-Me}}$Ala by dFx-1OMe, dFx-2OMe dFx-4OMe dFx-5OMe dFx-10OMe dFx-all OMe and dFx-part OMe. 18
Supplementary Figure 4. Acylation of microhelix with L-Ala by dFx-1OMe, dFx-2OMe dFx-4OMe dFx-5OMe dFx-10OMe dFx-all OMe and dFx-part OMe. 20
Supplementary Figure 5. Acylation of microhelix with $^{N\text{-Me}}$Ala by dFx- 1-3OMe dFx- 43-45OMe and dFx- 44-46-3OMe. 21
Supplementary Figure 6. Acylation of microhelix with L-Ala by dFx- 1-3OMe dFx- 43-45OMe and dFx- 44-46-3OMe. 22
Supplementary Figure 7. Acylation of microhelix with all the DBE substrates by dFx. 23
Supplementary Figure 8. The aminoacylation efficiency for dFx-3OMe, dFx-3F and dFx-3MOE with others acid-urea PAGE gel to derive standard deviations. 25
Supplementary Figure 9. The aminoacylation efficiency for eFx-3OMe and eFx with all acid-urea PAGE gel. 26
Supplementary Figure 10. The effect of Mg^{2+} concentration about the Substrate with DBE groups on the aminoacylation efficiency of dFx-3OMe. 27
Supplementary Figure 11. The effect of Mg^{2+} concentration about the Substrate with CME groups on the aminoacylation efficiency of eFx-3OMe. 28
Supplementary Figure 12.The nuclease resistance of the wild-type and modified flexizymes. 29
Supplementary Figure 13. The mass spectrum of the dFx. 30
Supplementary Figure 14. The mass spectrum of the dFx-3OMe. 30
Supplementary Figure 15. The mass spectrum of the dFx-3F. 31
Supplementary Figure 16. The mass spectrum of the dFx-3MOE. 31
Supplementary Figure 17. The mass spectrum of the dFx-1OMe. 32
Supplementary Figure 18. The mass spectrum of the dFx-2OMe. 32
Supplementary Figure 19. The mass spectrum of the dFx-4OMe. 33
Supplementary Figure 20. The mass spectrum of the dFx-5OMe. 33
Supplementary Figure 21. The mass spectrum of the dFx-10OMe. 34
Supplementary Figure 22. The mass spectrum of the dFx-all OMe. 34
Supplementary Figure 23. The mass spectrum of the dFx-part OMe. 35
Supplementary Figure 24. The mass spectrum of the dFx- 1-3OMe. 35
Supplementary Figure 25. The mass spectrum of the dFx- 43-45OMe. 36

Supplementary Figure 26. The mass spectrum of the dFx- 44-46OMe. 36
Supplementary Figure 27. The mass spectrum of the eFx. 37
Supplementary Figure 28. The mass spectrum of the eFx-3OMe...37
Supplementary Figure 29. The mass spectrum of the microhelix. 38
Supplementary Figure 30. ^{1}H NMR (600 MHz, DMSO-d_6) of **1**..39
Supplementary Figure 31. ^{13}C NMR (150 MHz, DMSO-d_6) of **1**...39
Supplementary Figure 32. The mass spectrum of **1**. 40
Supplementary Figure 33. ^{1}H NMR (600 MHz, MeOD) of **2**...41
Supplementary Figure 34. ^{13}C NMR (150 MHz, MeOD) of **2**...41
Supplementary Figure 35. The mass spectrum of **2**. 42
Supplementary Figure 36. ^{1}H NMR (600 MHz, MeOD) of **3**...43
Supplementary Figure 37. ^{13}C NMR (150 MHz, MeOD) of **3**...43
Supplementary Figure 38. The mass spectrum of **3**. 44
Supplementary Figure 39. ^{1}H NMR (600 MHz, MeOD) of **4**...45
Supplementary Figure 40. ^{13}C NMR (150 MHz, MeOD) of **4**...45
Supplementary Figure 41. The mass spectrum of **4**. 46
Supplementary Figure 42. ^{1}H NMR (600 MHz, MeOD) of **5**...47
Supplementary Figure 43. ^{13}C NMR (150 MHz, MeOD) of **5**...47
Supplementary Figure 44. The mass spectrum of **5**. 48
Supplementary Figure 45. ^{1}H NMR (500 MHz, MeOD) of **6**...49
Supplementary Figure 46. ^{13}C NMR (125 MHz, MeOD) of **6**...49
Supplementary Figure 47. The mass spectrum of **6**. 50
Supplementary Figure 48. ^{1}H NMR (500 MHz, MeOD) of **7**...51
Supplementary Figure 49. ^{13}C NMR (150 MHz, MeOD) of **7**...51
Supplementary Figure 50. The mass spectrum of **7**. 52
Supplementary Figure 51. ^{1}H NMR (600 MHz, MeOD) of **8**...53
Supplementary Figure 52. ^{13}C NMR (150 MHz, MeOD) of **8**...53
Supplementary Figure 53. The mass spectrum of **8**. 54
Supplementary Figure 54. ^{1}H NMR (600 MHz, DMSO-d_6) of **9**..55
Supplementary Figure 55. ^{13}C NMR (150 MHz, DMSO-d_6) of **9**...55
Supplementary Figure 56. The mass spectrum of **9**. 56
Supplementary Figure 57. ^{1}H NMR (600 MHz, DMSO-d_6) of **10**..57
Supplementary Figure 58. ^{13}C NMR (150 MHz, DMSO-d_6) of **10**.......................................57
Supplementary Figure 59. The mass spectrum of **10**. 58
Supplementary Figure 60. ^{1}H NMR (600 MHz, DMSO-d_6) of **11**..59
Supplementary Figure 61. ^{13}C NMR (150 MHz, DMSO-d_6) of **11**.......................................59
Supplementary Figure 62. The mass spectrum of **11**. 60
Supplementary Figure 63. ^{1}H NMR (600 MHz, MeOD) of **12**...61
Supplementary Figure 64. ^{13}C NMR (150 MHz, MeOD) of **12**...61
Supplementary Figure 65. The mass spectrum of **12**. 62
Supplementary Figure 66. ^{1}H NMR (600 MHz, MeOD) of **13**...63
Supplementary Figure 67. ^{13}C NMR (150 MHz, MeOD) of **13**...63
Supplementary Figure 68. The mass spectrum of **13**. 64
Supplementary Figure 69. ^{1}H NMR (500 MHz, MeOD) of **14**...65

Supplementary Figure 70. ^{13}C NMR (150 MHz, MeOD) of **14**..65
Supplementary Figure 71. The mass spectrum of **14**. 66
Supplementary Figure 72. ^{1}H NMR (600 MHz, $CDCl_3$) of **15**. 67
Supplementary Figure 73. ^{13}C NMR (150 MHz, $CDCl_3$) of **15**. 67
Supplementary Figure 74. The mass spectrum of **15**. 68
Supplementary Figure 75. ^{1}H NMR (600 MHz, DMSO-d_6) of **16**..69
Supplementary Figure 76. ^{13}C NMR (150 MHz, DMSO-d_6) of **16**..69
Supplementary Figure 77. The mass spectrum of **16**. 70
Supplementary Figure 78. ^{1}H NMR (600 MHz, $CDCl_3$) of **17**. 71
Supplementary Figure 79. ^{13}C NMR (150 MHz, $CDCl_3$) of **17**. 71
Supplementary Figure 80. The mass spectrum of **17**. 72
Supplementary Figure 81. ^{1}H NMR (600 MHz, $CDCl_3$) of **18**. 73
Supplementary Figure 82. ^{13}C NMR (150 MHz, $CDCl_3$) of **18**. 73
Supplementary Figure 83. The mass spectrum of **18**. 74
Reference 75

Supplementary Methods

Materials and Methods

All acid substrates were synthesized from the corresponding *N*-Boc protected amino acids. All CME substrates were synthesized same procedure as previously described. All DBE substrates were synthesized by coupling with 3,5-dinitrobenzylchloride. NMR spectra were recorded on a Bruker DXR-600 instrument (600 MHz for ^{1}H, 150 MHz for ^{13}C, respectively) equipped with a 5 mm BBO Prodigy cryoprobe, (Bruker Instruments Inc., Germany) or on a Bruker AMX 500 (500 MHz for ^{1}H,125 MHz for, respectively). ESI-MS was recorded on a Thermo Scientific™ *Q*-Exactive LC-MS system.

General procedure for synthesis of DBE substrates

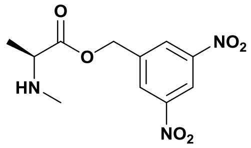
A mixture of *N*-Boc-Amino Acid (0.5 mmol, 1 eq), triethylamine (1.0 mmol, 2 eq) and 3,5-dinitrobenzyl chloride (0.5 mmol, 1 eq) in 0.1 mL of dimethylformamide was stirred at room temperature for 12 h. After the reaction, diethylether (9 mL) was added and the solution was washed with 0.5 M HCl (3 mL x 3), 4 % $NaHCO_3$ (3 mL x 3) and brine (5 mL x1), and the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product is purified by column chromatography. Fractions of interest were combined and the solvents were removed under reduced pressure yielding as intermediate products. The intermediate products were dissolved in 2 mL of 4 M HCl/ethylacetate and incubated for 20 min at room temperature. The solution was concentrated under reduced pressure and the product was precipitated by the addition of diethylether (3 mL).

General procedure for synthesis of CME substrates

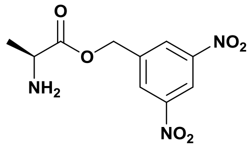
A mixture of triethylamine (1.5 mmol, 1 eq) and chloroacetonitrile (2.0 mmol, 2 eq) is cooled in an ice-water bath while *N*-Boc-Amino Acid (1.0 mmol, 1 eq) is added, in small portions, with stirring. The addition of the protected amino acid requires about

15 min. Stirring and cooling are continued for about a half hour and the mixture is stored at room temperature overnight. The thick mass is diluted with ethyl acetate, and the insoluble material (triethylammonium chloride) is removed by filtration and washed with ethyl acetate. The solution is extracted with 0.5 N HCI, 0.5 $NaKHCO_3$, and water, dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo.* The solvent was removed under reduced pressure to obtain the crude product. The crude product is purified by column chromatography. Fractions of interest were combined and the solvents were removed under reduced pressure yielding as final product.

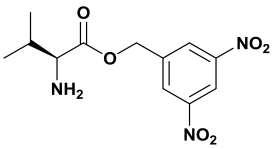
Characterizations of substrates



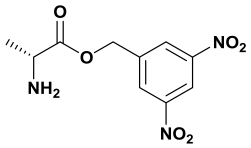
***N*-Methyl-*L*-alanine-3,5-dinitrobenzyl ester (1).** ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.82 (t, J = 2.2 Hz, 1H), 8.74 (d, J = 2.1 Hz, 2H),5.54 – 5.46 (m, 2H), 4.23 (q, J = 7.1 Hz, 1H), 2.61 (s, 3H), 1.47 (d, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 169.37, 148.15, 139.59, 128.59, 118.51, 65.15, 55.26, 30.72, 14.07; HRMS(ESI): Exact mass calcd for $C_{11}H_{14}N_3O_6$ $[M+H]^+$ 284.0883, found 284.0875.



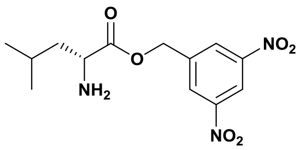
***L*-Alanine-3,5-dinitrobenzyl ester (2).**^{1}H NMR (600 MHz, MeOD) δ: 8.98 (t, J = 2.1 Hz, 1H), 8.71 (d, J = 2.1 Hz, 2H), 5.54 (d, J = 3.4 Hz, 2H), 4.28 (q, J = 7.2 Hz, 1H), 1.62 (d, J = 7.2 Hz, 3 H); ^{13}C NMR (150 MHz, MeOD) δ: 170.78, 150.04, 141.03, 129.41, 119.55, 66.77, 4 9.86, 16.17; HRMS (ESI): Exact mass calcd for $C_{10}H_{12}N_3O_6$ $[M+H]^+$ 270.0726, found 270.0719.



***L*-Valine-3,5-dinitrobenzyl ester (3).** ^{1}H NMR (600 MHz, MeOD) δ: 8.99 (t, J = 2.1 Hz, 1H), 8.73 (d, J = 2.0 Hz, 2H), 5.56 (q, J = 13.1 Hz, 2H), 4.11 (d, J = 4.6 Hz, 1H), 2.38 (heptd, J = 7.0, 4.6 Hz, 1H), 1.10 (d, J = 1.4 Hz, 3H), 1.09 (d, J = 1.4 Hz, 3H);^{13}C NMR (150 MHz, MeOD) δ: 169.87, 150.03, 140.93, 129.57, 119.62, 66.75, 59.38, 31.05, 18.33; HRMS (ESI): Exact mass calcd for $C_{12}H_{16}N_3O_6$ $[M+H]^+$ 298.1039, found 298.1031.

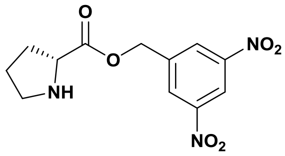


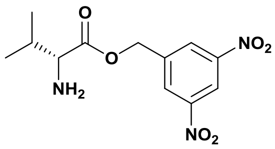
***D*-Alanine-3,5-dinitrobenzyl ester (4).** ^{1}H NMR (600 MHz, MeOD) δ: 8.99 (t, J = 2.1 Hz, 1H), 8.71 (d, J = 2.1 Hz, 2H), 5.53 (d, J = 3.4 Hz, 2H), 4.27 (q, J = 7.2 Hz, 1H), 1.61 (d, J = 7.2 Hz, 3 H); ^{13}C NMR (150 MHz, MeOD), δ: 170.81, 150.07, 141.00, 129.37, 119.58, 66.77, 49.84, 16.16; HRMS (ESI): Exact mass calcd for $C_{10}H_{12}N_3O_6$ $[M+H]^+$ 270.0726, found 270.0719.



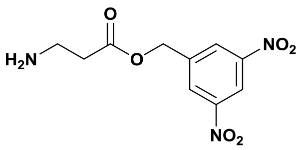
***D*-Leucine-3,5-dinitrobenzyl ester (5).** ^{1}H NMR (600 MHz, MeOD) δ: 8.97 (t, J = 2.1 Hz, 1H), 8.72 (d, J = 2.1 Hz, 2H), 5.55 (d, J = 4.2 Hz, 2H), 4.21 (t, J = 7.1 Hz, 1H), 1.89 (dd, J = 13.5, 6.8 Hz, 1H), 1.83 (dq, J = 13.1, 6.4 Hz, 1H), 1.77 (dt, J = 13.7, 7.1 Hz, 1H), 1.03 (d, J = 6.4 Hz, 3H), 1.01 (d, J = 6.3 Hz, 3H); ^{13}C NMR (150 MHz, MeOD) δ:170.76, 149.98, 140.98, 129.43, 119.51, 66.77, 52.49, 40.65, 25.68, 22.50, 22.36; HRMS(ESI): Exact mass calcd for $C_{13}H_{18}N_3O_6$ $[M+H]^+$ 312.1196, found 312.1188.

***D*-Proline-3,5-dinitrobenzyl ester (6).** ^{1}H NMR (500 MHz, MeOD) δ: 8.99 (t, J = 2.1 Hz, 1H), 8.72 (d, J = 2.1 Hz, 2H), 5.54 (d, J = 3.1 Hz, 2H), 4.59 (dd, J = 8.7, 7.3 Hz, 1H), 3.48 – 3.37 (m, 2H), 2.53 – 2.45 (m, 1H), 2.25 – 2.18 (m, 1H), 2.13 – 2.07 (m, 2H); ^{13}C NMR (125 MHz, MeOD) δ:169.89, 150.06, 140.85, 129.49, 119.61, 60.69, 47.28, 29.33, 24.57; HRMS (ESI): Exact mass calcd for $C_{12}H_{14}N_3O_6$ $[M+H]^+$ 296.0883,found 296.0875.

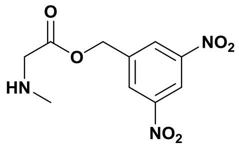




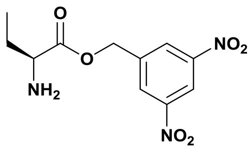
***D*-Valine-3,5-dinitrobenzyl ester (7).** ^{1}H NMR (500 MHz, MeOD) δ: 8.98 (t, J = 2.1 Hz, 1H), 8.73 (d, J = 2.1 Hz, 2H), 5.56 (q, J = 13.1 Hz, 2H), 4.11 (d, J = 4.6 Hz, 1H), 2.38 (pd, J = 6.9, 4.5 Hz, 1H), 1.09 (d, J = 6.9 Hz, 6H); ^{13}C NMR (150 MHz, MeOD) δ:169.85, 150.02, 140.92, 129.57, 119.60, 66.75, 59.38, 31.04, 18.33; HRMS(ESI): Exact mass calcd for $C_{12}H_{16}N_3O_6$ $[M+H]^+$ 298.1039, found 298.1030.



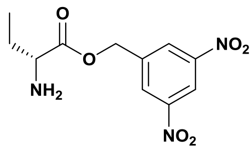
***β*-Alanine-3,5-dinitrobenzyl ester (8).** ^{1}H NMR (600 MHz, MeOD) δ: 8.97 (t, J = 2.2 Hz, 1H), 8.68 (d, J = 2.1 Hz, 2H), 5.43 (s, 2H), 3.25 (d, J = 6.5 Hz, 2H), 2.89 (t, J = 6.5 Hz, 2H); ^{13}C NMR (150 MHz, MeOD) δ: 171.79, 150.03, 141.72, 129.26, 119.33, 65.77, 36.27, 32.15; HRMS (ESI): Exact mass calcd. for $C_{10}H_{12}N_3O_6$ $[M+H]^+$ 270.0726, found 270.0718.



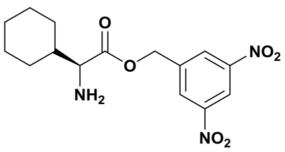
N-Methyl -glycine (Sarcosine)-3,5-dinitrobenzyl ester (9). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.80 (t, J = 2.2 Hz, 1H), 8.75 (d, J = 2.1 Hz, 2H), 5.50 (s, 2H), 4.09 (s, 2H), 2.58 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ:166.55, 148.12, 139.71, 128.57, 118.43, 64.81, 47.96, 32.57; HRMS(ESI): Exact mass calcd for $C_{10}H_{12}N_3O_6$ $[M+H]^+$ 270.0726, found 270.0719.



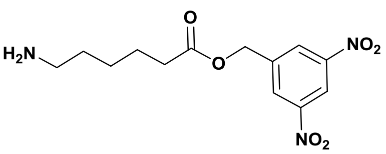
***L*-2-Aminobutyric acid-3,5-dinitrobenzyl ester (10).** ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.80 (d, J = 2.1 Hz, 1H), 8.75 (d, J = 2.1 Hz, 2H), 5.50 (s, 2H), 4.13 – 4.02 (m, 1H), 1.94 – 1.88 (m, 2H), 0.95 (t, J = 7.5 Hz, 3H);^{13}C NMR (150 MHz, DMSO-d_6) δ:169.16, 148.12, 139.73, 128.57, 118.45, 64.96, 53.06, 23.40, 9.26; HRMS (ESI): Exact mass calcd for $C_{11}H_{14}N_3O_6$ $[M+H]^+$ 284.0883, found 284.0875.



D-2-Aminobutyric acid-3,5-dinitrobenzyl ester (11). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.81 (t, J = 2.1 Hz, 1H), 8.75 (d, J = 2.1 Hz, 2H), 5.51 (s, 2H), 4.10 (d, J = 6.1 Hz, 1H), 1.92 – 1.89 (m, 2H), 0.95 (t, J = 7.5 Hz, 3H);^{13}C NMR (150 MHz, DMSO-d_6) δ: 169.15, 148.12, 139.70, 128.56, 118.46, 64.97, 53.03, 23.39, 9.24; HRMS(ESI): Exact mass calcd for $C_{11}H_{14}N_3O_6$ $[M+H]^+$ 284.0883, found 284.0874.

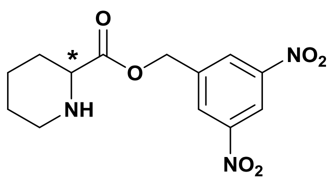


***L*-Cyclohexyl glycine-3,5-dinitrobenzyl ester (12).** ^{1}H NMR (600 MHz, MeOD) δ: 9.00 (t, J = 2.1 Hz, 1H), 8.71 (d, J = 2.1 Hz, 2H), 5.55 (s, 2H), 4.06 (d, J = 4.8 Hz, 1H), 2.01 (tdt, J = 11.6, 4.8, 3.2 Hz, 1H), 1.87 – 1.77 (m, 3H), 1.76 – 1.64 (m, 2H), 1.39 – 1.07 (m, 5H);^{13}C NMR (150 MHz, MeOD) δ: 169.92, 150.08, 141.00, 129.45, 119.64, 66.70, 58.86, 40.62, 29.66, 29.42, 26.94 (d, J = 6.6 Hz), 26.70; HRMS (ESI):Exact mass calcd for $C_{15}H_{20}N_3O_6$ $[M+H]^+$ 338.1352, found 338.1343.

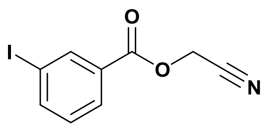


Aminocaproic acid-3,5-dinitrobenzyl ester (13). ^{1}H NMR (600 MHz, MeOD) δ: 8.95 (t, J = 2.1 Hz, 1H), 8.63 (d, J = 2.1 Hz, 2H), 5.36 (s, 2H), 2.93 (t, J = 7.7 Hz, 2H), 2.52 (t, J = 7.3 Hz, 2H), 1.77 – 1.65 (m, 4H), 1.46 (tt, J = 10.1, 6.5 Hz, 2H);^{13}C NMR (150 MHz, MeOD)

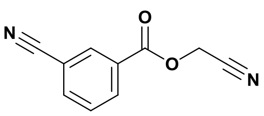
δ:174.39, 150.00, 142.47, 128.85, 119.09, 65.05, 40.54, 34.38, 28.27, 26.89, 25.35; HRMS (ESI): Exact mass calcd for $C_{13}H_{18}N_3O_6$ $[M+H]^+$ 312.1196, found 312.1188.



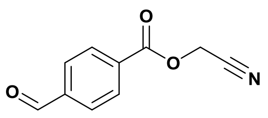
(±)-Pipecolic acid-3,5-dinitrobenzyl ester (14). ^{1}H NMR (500 MHz, MeOD) δ: 8.99 (t, J = 2.1 Hz, 1H), 8.70 (d, J = 2.1 Hz, 2H), 5.54 (d, J = 2.3 Hz, 2H), 4.20 (dd, J = 11.8, 3.5 Hz, 1H), 3.47 – 3.43 (m, 1H), 3.07 (td, J = 12.5, 3.5 Hz, 1H), 2.38 – 2.33 (m, 1H), 1.95 – 1.88 (m, 2H), 1.83 – 1.76 (m, 1H), 1.70 (ddt, J = 10.2, 8.6, 2.5 Hz, 2H);^{13}C NMR (150 MHz, MeOD) δ: 169.77, 150.08, 140.89, 129.39, 119.62, 66.82, 57.86, 45.26, 27.16, 22.83, 22.74. HRMS (ESI): Exact mass calcd for $C_{13}H_{16}N_3O_6$ $[M+H]^+$ 310.1034, found 310.1033.



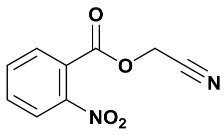
Cyanomethyl 3-iodobenzoate (15). ^{1}H NMR (600 MHz, $CDCl_3$) δ:8.33 (t, J = 1.7 Hz, 1H), 7.98 (dt, J = 7.8, 1.4 Hz, 1H), 7.91 (dt, J = 8.0, 1.5 Hz, 1H), 7.19 (t, J = 7.8 Hz, 1H), 4.95 (s, 2H);^{13}C NMR (150 MHz, $CDCl_3$)δ:163.50, 142.89, 138.67, 130.35, 129.64, 129.10, 114.33, 93.98, 49.15; HRMS (ESI): Exact mass calcd for $C_9H_6INO_2Na$ $[M+Na]^+$ 309.9341, found 309.9336.



Cyanomethyl 3-cyanobenzoate (16). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.39 (t, J = 1.7 Hz, 1H), 8.28 (dt, J = 8.1, 1.4 Hz, 1H), 8.19 (dt, J = 7.7, 1.5 Hz, 1H), 7.79 (t, J = 7.9 Hz, 1H), 5.26 (s, 2H)； ^{13}C NMR (150 MHz, DMSO-d_6)δ:163.34, 137.55, 134.00, 133.18, 130.51, 129.27, 117.79, 115.85, 112.39, 50.33.; HRMS (ESI): Exact mass calcd for $C_{10}H_5N_2O_2$ $[M-H]^-$ 185.0357, found 185.0359.



Cyanomethyl 4-formylbenzoate (17). ^{1}H NMR (600 MHz, $CDCl_3$) δ:10.05 (s, 1H), 8.17 – 8.11 (m, 2H), 7.95 – 7.89 (m, 2H), 5.00 (s, 2H).^{13}C NMR (150 MHz, $CDCl_3$)δ:191.48, 164.12, 139.93, 132.73, 130.72, 129.77, 114.26, 49.36; HRMS (ESI): Exact mass calcd for $C_{10}H_8NO_3$ $[M+H]^+$ 190.0504, found 190.0498.



Cyanomethyl 2-nitrobenzoate (18). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.01 – 7.97 (m, 1H), 7.75 – 7.73 (m, 2H), 7.73 – 7.69 (m, 1H), 4.96 (s, 2H). ^{13}C NMR (150 MHz, CDCl3) δ:164.25, 147.81, 133.63, 132.85, 130.00, 125.71, 124.47, 113.75, 49.87; HRMS (ESI): Exact mass calcd for $C_9H_6N_2O_4Na$ $[M+Na]^+$ 229.0225, found 229.0220.

The synthesis of the chemically modified RNAs

All chemical modifications of RNA are ordered through Shanghai Primerna NAT Co.,Ltd.

Acylation of microhelix

The acylation reaction with the modified flexizymes was carried out as follows: 1 μL of 10 μM microhelix, 3 μL of RNase-free water, and 1 μL of 0.5 M HEPES-KOH (pH=7.5, 8.5, or 9.5) were added separately to a 0.2 mL microcentrifuge tube with 1 μL of 10 μM the modified flexizymes. The mixture was then heated at 95°C for 3 min and subsequently cooled down to 25°C over 20 min. 2 μL of 0.3 M $MgCl_2$ was added to the mixture and incubated for over 5 min at room temperature and the reaction was on ice for 5 min. Finally, 2 μL of 25 mM or 100 mM activated esters dissolved in DMSO was added to the pre-existing mixture. The reaction mixture was allowed to proceed on ice within 2-120 h (2, 6, 24, 48, 72 and 120h). ($^{N\text{-Me}}$Ala and L-Ala were acylated under 25 mM at pH 7.5 with 6 and 2 h reaction times)

To explore the effect of the concentration of Mg^{2+} on the catalytic efficiency of Fx, we used microhelix acylate substrate **2** and **9** or **15-17** under different conditions by dFx-3OMe or eFx-3OMe, whose concentration of 2 μL of 0.3 M $MgCl_2$ changed to 0.1 M and 0.2 M or 0.2 M, 0.4 M, 0.5 M, and 0.6 M. These results have shown in Figure S10-S11.

The analysis of Acid-Urea PAGE

At the designated time points, 2 μL of 2×RNA Loading Buffer (comprising 0.15 mM NaOAc, pH=5.2, 0.5 M EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol FF, and 93% formamide) were used to extract 2 μL from the aforementioned reaction mixture, effectively stopping the acylation reaction. The resulting reaction mixture, which did not require ethanol precipitation, was loaded onto a 20% acid-urea PAGE gel, using 50 mM NaOAc (pH=5.2) as the running buffer. The acid-urea PAGE gel was run at 180V on ice for 2 h. Subsequently, GelRed (Biosharp) staining was performed for 10 min, and the gel was visualized on a Gel Doc XR+ (Bio-Rad).

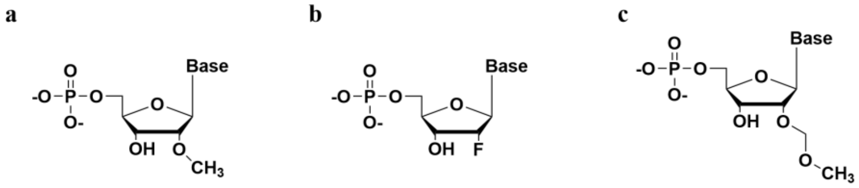
The aminoacylation efficiency of the ribozymes was calculated based on grayscale values using ImageJ, following the formula:

Aminoacylation Efficiency % = (Grayscale value of (mh+AA)) / (Grayscale value of mh) + (Grayscale value of (mh+AA)) × 100%.

Table S1. The names, sequences, and structures of the involved RNAs

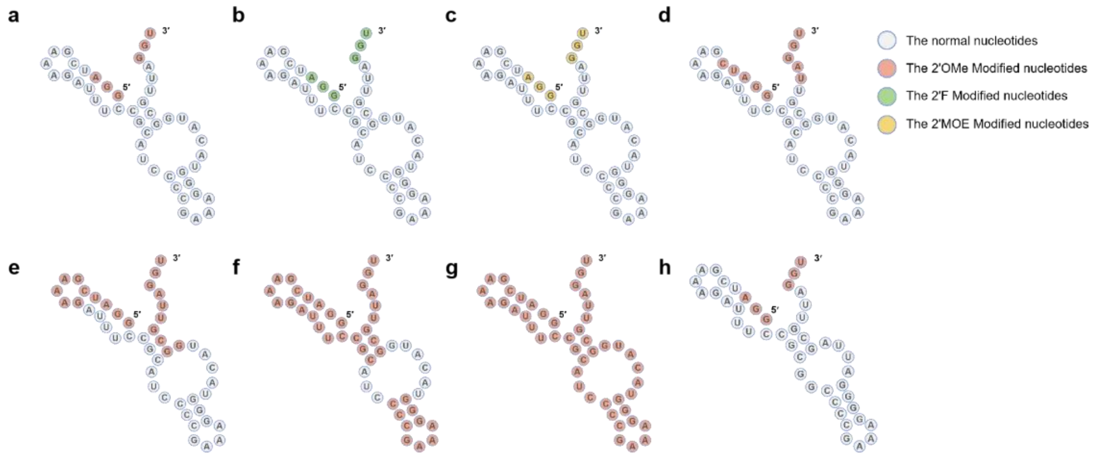
	Sense (5'-3')	remark
microhelix	GG CUCUG UUCGC AGAGC CGCCA	
dFx	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	
dFx-3OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of three nucleotides at 5' and 3' ends
dFx-3F	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Fluorine substitution of three nucleotides at 5' and 3' ends
dFx-3MOE	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-MOE of three nucleotides at 5' and 3' ends
dFx-1OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of one nucleotides at 5' and 3' ends
dFx-2OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of two nucleotides at 5' and 3' ends
dFx-4OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of four nucleotides at 5' and 3' ends
dFx-5OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of nucleotides at 5' and 3' ends
dFx-10OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of ten nucleotides at 5' and 3' ends
dFx-part OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of 1-18, 22-31, 38-46 nucleotides
dFx-all OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of all of nucleotides
dFx- 1-3OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of 1-3 nucleotides at 5' ends
dFx- 43-45OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of 43-45 nucleotides at 5' ends
dFx- 44-46OMe	GG AUCGAAAGAU UUCCGCAUCC CCGAAAGGGU ACAUGGCGUU AGGU	2'-Methylation modification of 44-46 nucleotides at 5' ends
eFx	GG AUCGAAAGAU UUCCGCGGCC CCGAAAGGGG AUUAGCGUUA GGU	
eFx-3OMe	GG AUCGAAAGAU UUCCGCGGCC CCGAAAGGGG AUUAGCGUUA GGU	2'-Methylation modification of three nucleotides at 5' and 3' ends

Note: The red letters present where the nucleotides were modified.



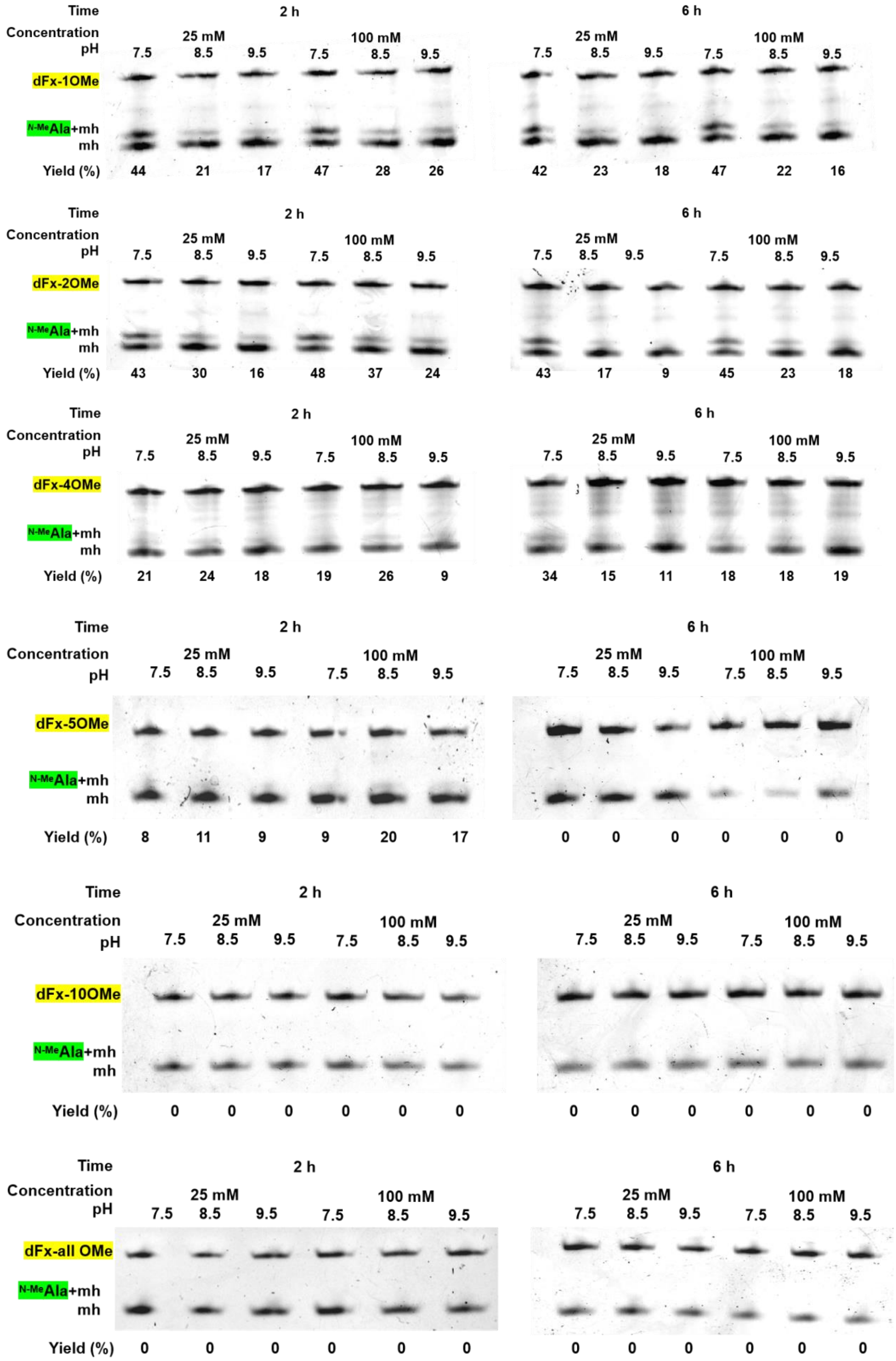
Supplementary Figure 1. The chemical modification methods for nucleosides.

(a) The 2'-OMe modified structure of nucleoside.
(b) The 2'-F modified structure of nucleoside.
(c) The 2'-MOE modified structure of nucleoside.

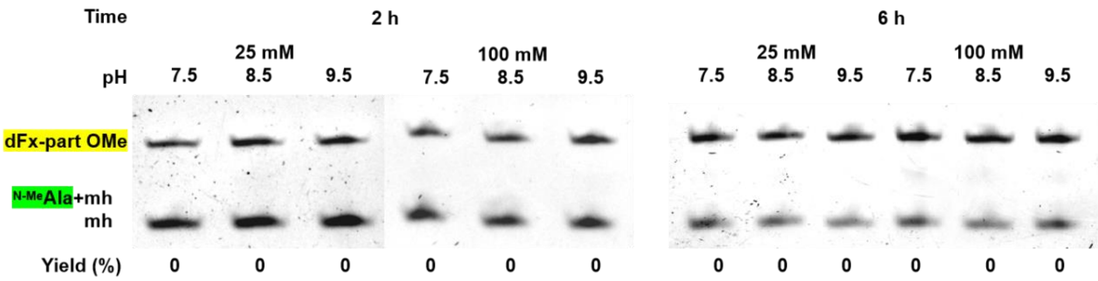


Supplementary Figure 2. The chemical modification methods for Fx.

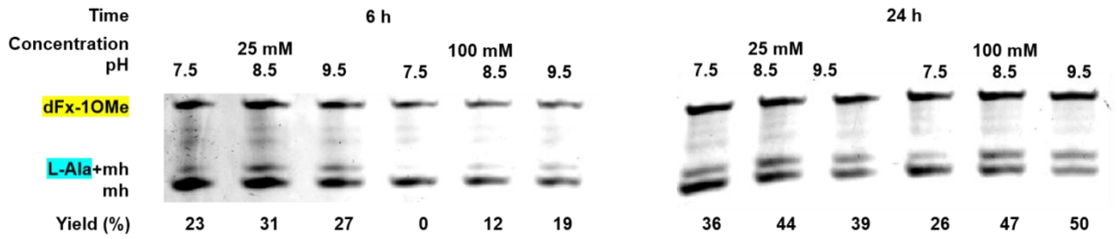
(a) The secondary structure of dFx-3OMe.
(b) The secondary structure of dFx-3F.
(c) The secondary structure of dFx-3MOE.
(d) The secondary structure of dFx-5OMe.
(e) The secondary structure of dFx-10OMe.
(f) The secondary structure of dFx-part OMe.
(g) The secondary structure of dFx-all OMe.
(h) The secondary structure of eFx-3OMe.

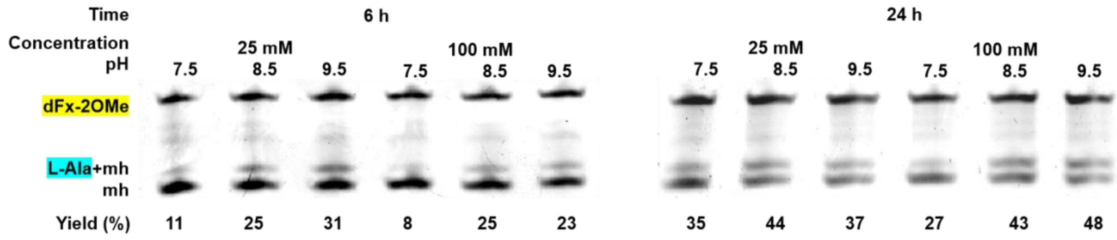


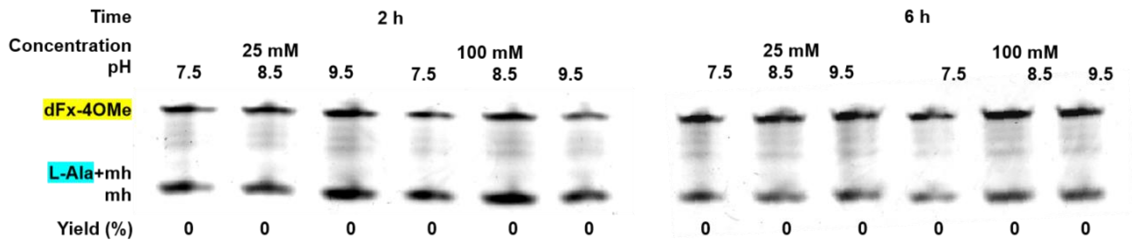
Time
2 h
6 h
Concentration
25 mM
100 mM
pH
7.5 8.5 9.5 7.5 8.5 9.5
dFx-1OMe
N-MeAla+mh
mh
Yield (%)
44 21 17 47 28 26
42 23 18 47 22 16
dFx-2OMe
43 30 16 48 37 24
43 17 9 45 23 18
dFx-4OMe
21 24 18 19 26 9
34 15 11 18 18 19
dFx-5OMe
8 11 9 9 20 17
0 0 0 0 0 0
dFx-10OMe
0 0 0 0 0 0
0 0 0 0 0 0
dFx-all OMe
0 0 0 0 0 0
0 0 0 0 0 0

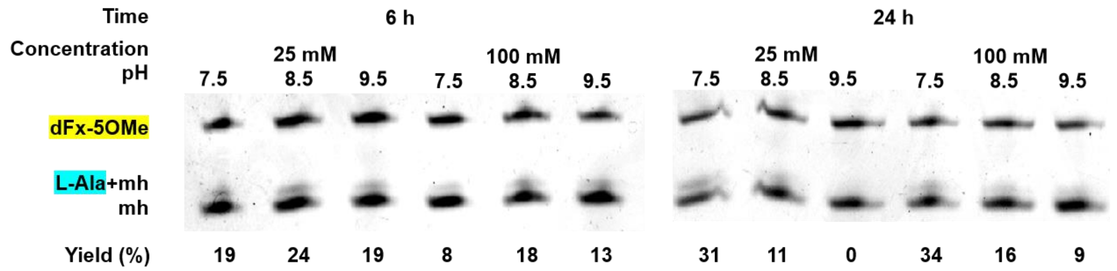


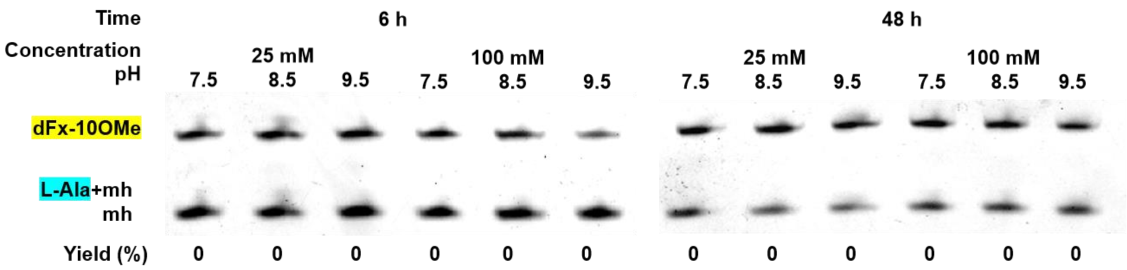
Supplementary Figure 3. Acylation of microhelix with $^{N\text{-Me}}$Ala by dFx-1OMe, dFx-2OMe dFx-4OMe dFx-5OMe dFx-10OMe dFx-all OMe and dFx-part OMe.

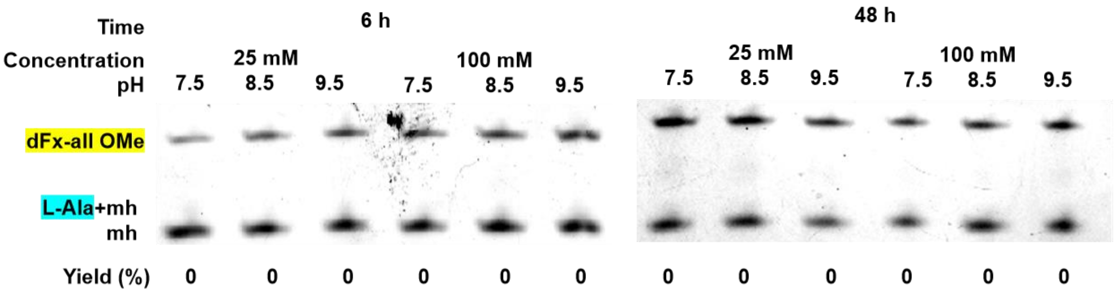

Time
6 h
24 h
Concentration
25 mM
100 mM
25 mM
100 mM
pH
7.5 8.5 9.5 7.5 8.5 9.5
7.5 8.5 9.5 7.5 8.5 9.5
dFx-1OMe
L-Ala+mh
mh
Yield (%)
23 31 27 0 12 19
36 44 39 26 47 50

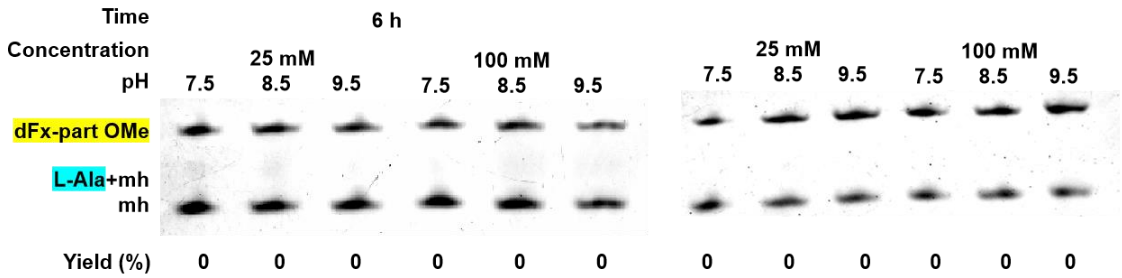

Time
6 h
24 h
Concentration
25 mM
100 mM
25 mM
100 mM
pH
7.5 8.5 9.5 7.5 8.5 9.5
7.5 8.5 9.5 7.5 8.5 9.5
dFx-2OMe
L-Ala+mh
mh
Yield (%)
11 25 31 8 25 23
35 44 37 27 43 48


Time
2 h
6 h
Concentration
25 mM
100 mM
25 mM
100 mM
pH
7.5 8.5 9.5 7.5 8.5 9.5
7.5 8.5 9.5 7.5 8.5 9.5
dFx-4OMe
L-Ala+mh
mh
Yield (%)
0 0 0 0 0 0
0 0 0 0 0 0

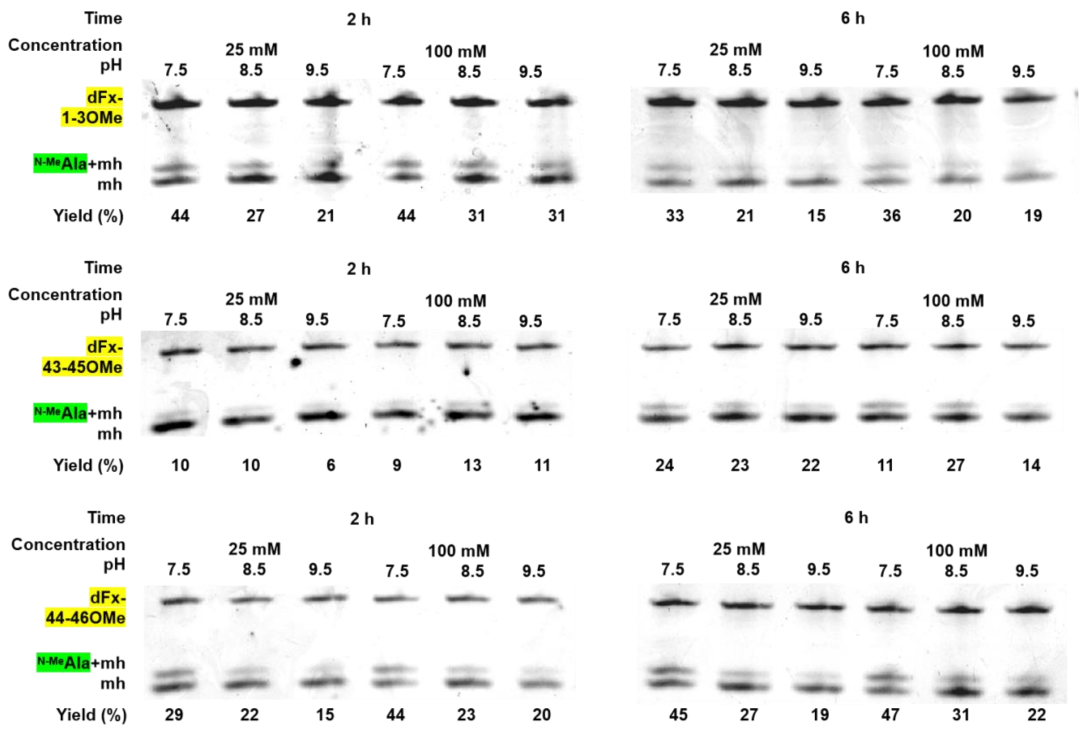

Time
6 h
24 h
Concentration
25 mM
100 mM
25 mM
100 mM
pH
7.5 8.5 9.5 7.5 8.5 9.5
7.5 8.5 9.5 7.5 8.5 9.5
dFx-5OMe
L-Ala+mh
mh
Yield (%)
19 24 19 8 18 13
31 11 0 34 16 9


Time
6 h
48 h
Concentration
25 mM
100 mM
25 mM
100 mM
pH
7.5 8.5 9.5 7.5 8.5 9.5
7.5 8.5 9.5 7.5 8.5 9.5
dFx-10OMe
L-Ala+mh
mh
Yield (%)
0 0 0 0 0 0
0 0 0 0 0 0

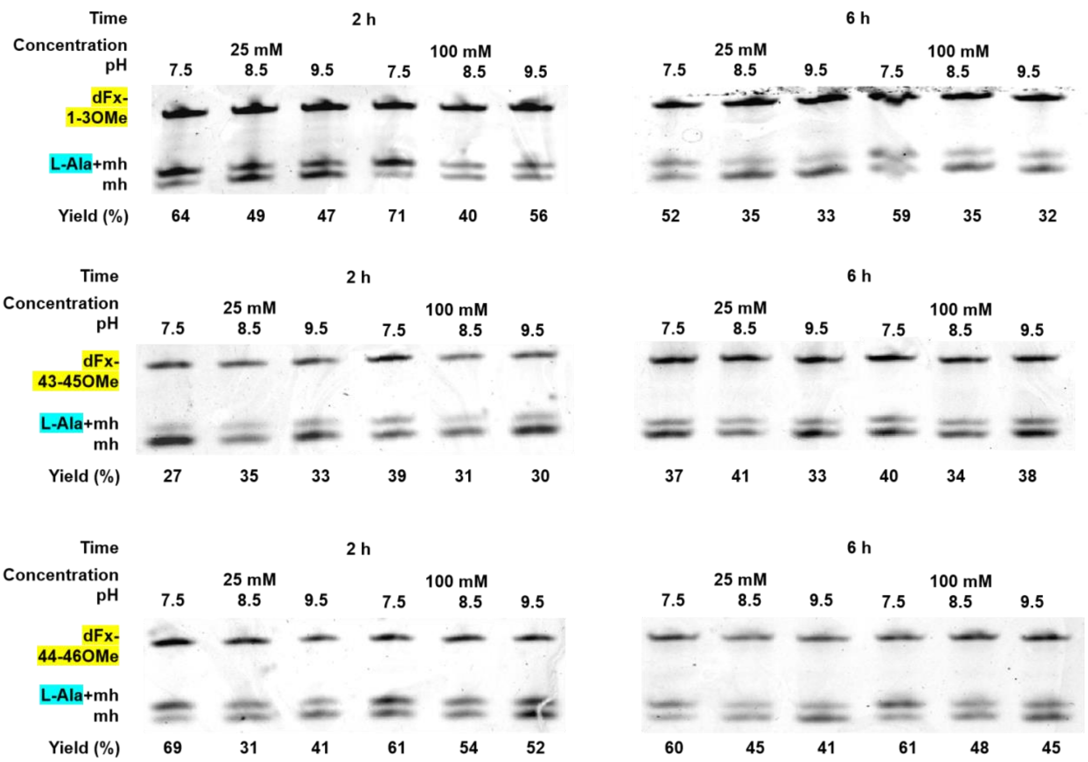

Time
6 h
48 h
Concentration
25 mM
100 mM
25 mM
100 mM
pH
7.5 8.5 9.5 7.5 8.5 9.5
7.5 8.5 9.5 7.5 8.5 9.5
dFx-all OMe
L-Ala+mh
mh
Yield (%)
0 0 0 0 0 0
0 0 0 0 0 0



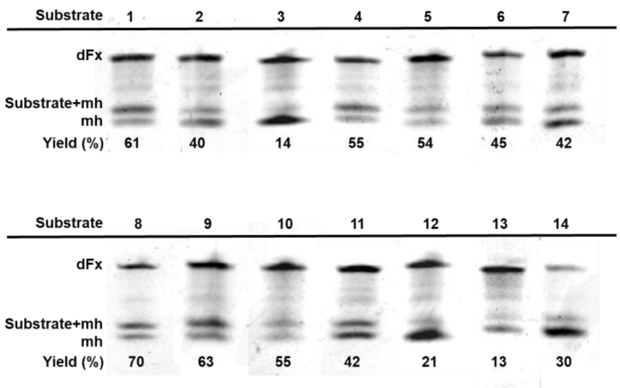
Supplementary Figure 4. Acylation of microhelix with L-Ala by dFx-1OMe, dFx-2OMe dFx-4OMe dFx-5OMe dFx-10OMe dFx-all OMe and dFx-part OMe.



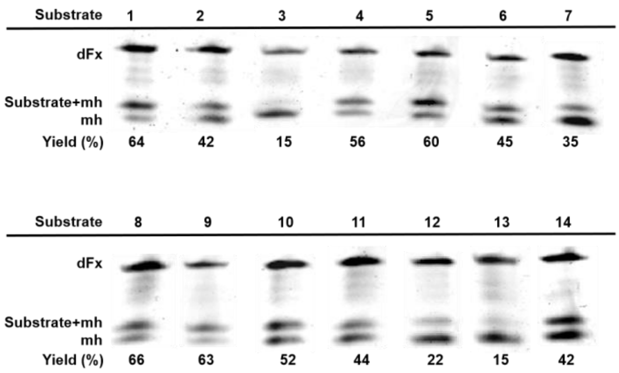
Supplementary Figure 5. Acylation of microhelix with $^{N\text{-}Me}$Ala by dFx- 1-3OMe dFx- 43-45OMe and dFx- 44-46-3OMe.



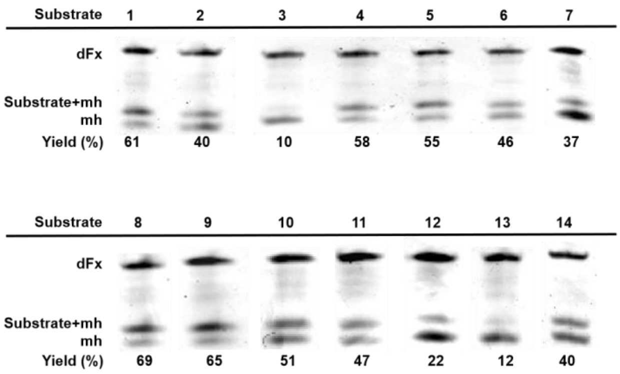
Supplementary Figure 6. Acylation of microhelix with L-Ala by dFx- 1-3OMe dFx- 43-45OMe and dFx- 44-46-3OMe.



Data **1** of aminoacylation efficiency of the wild-type dFx



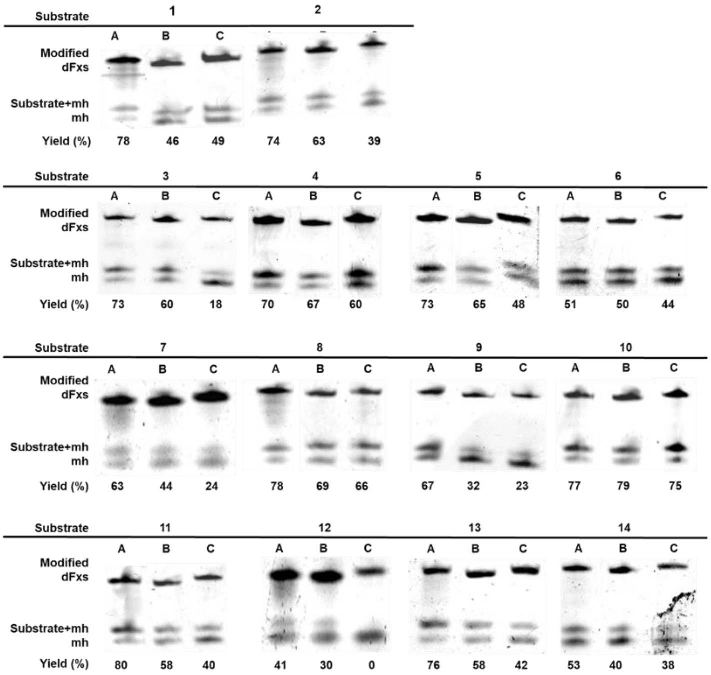
Data **2** of aminoacylation efficiency of the wild-type dFx



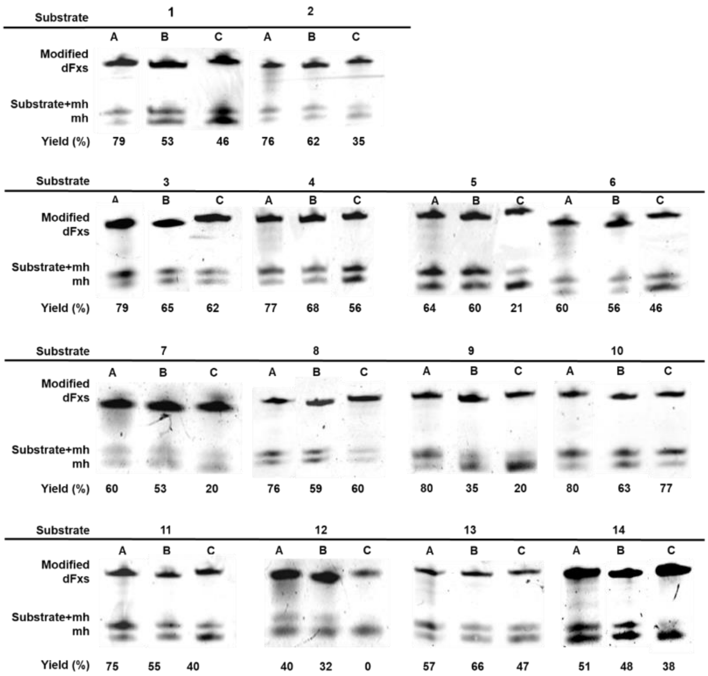
Data **3** of aminoacylation efficiency of the wild-type dFx

Supplementary Figure 7. Acylation of microhelix with all the DBE substrates by dFx.

NOTE: Compared to the literature reports, substrates **4**, **6**, and **8** have some discrepancies which exhibited efficiency enhancements of 16%, an increase of 17% and a decrease of 7%, respectively. However, the outcome shows that the modified Fx demonstrates higher catalytic activity than the unmodified. The others are within-range errors. It is worth mentioning that we used the chemically synthetic dFx rather than the dFx produced by transcription in vitro, in which the 5 'end has no phosphate group.



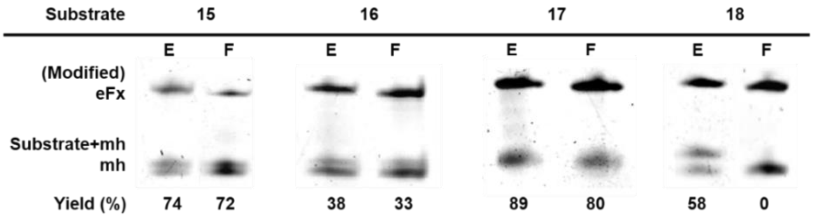
Data **2** of aminoacylation efficiency of the modified dFxs



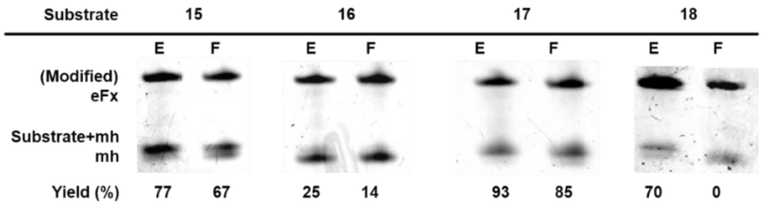
Data **3** of aminoacylation efficiency of the modified dFxs

Supplementary Figure 8. The aminoacylation efficiency for dFx-3OMe, dFx-3F and dFx-3MOE for standard deviations. To validate the reliability of the data, two additional experiments were conducted under these conditions. The standard deviation for Fig.2b is calculated based on these three sets of results. A represents dFx-3OMe, B represents dFx-3F and C represents dFx-3MOE.

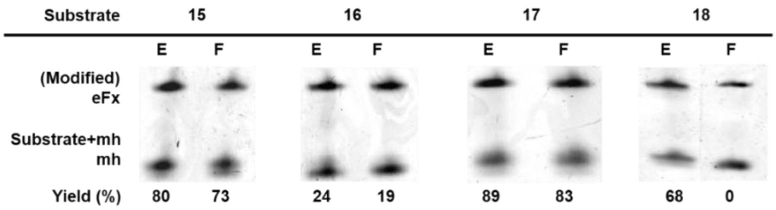
(a)



Data **1** of aminoacylation efficiency of the modified eFx-3OMe and eFx

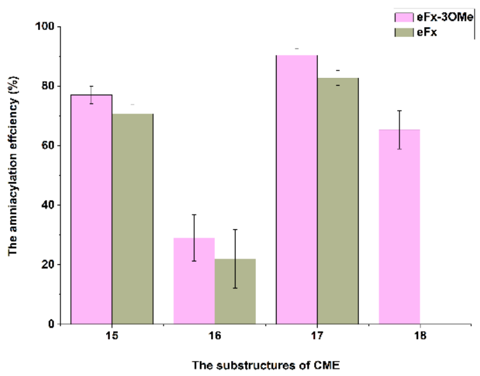


Data **2** of aminoacylation efficiency of the modified eFx-3OMe and eFx



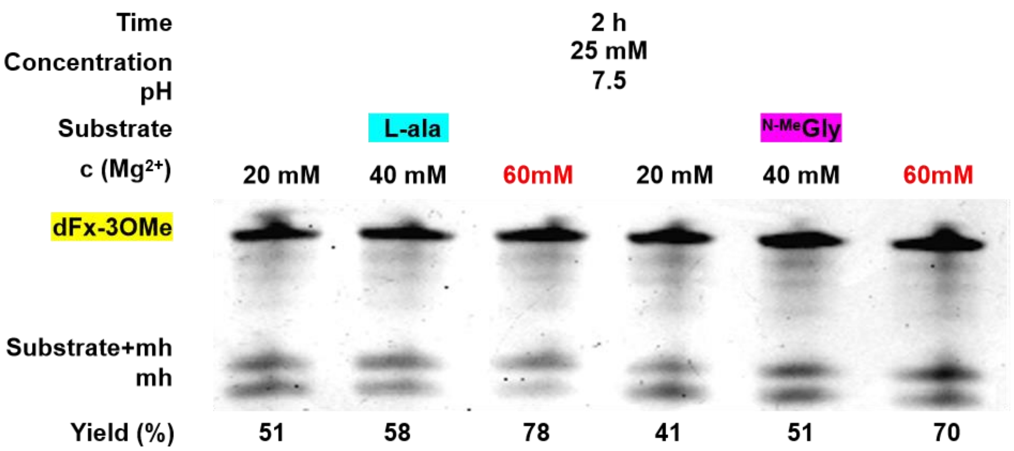
Data **3** of aminoacylation efficiency of the modified eFx-3OMe and eFx

(b)

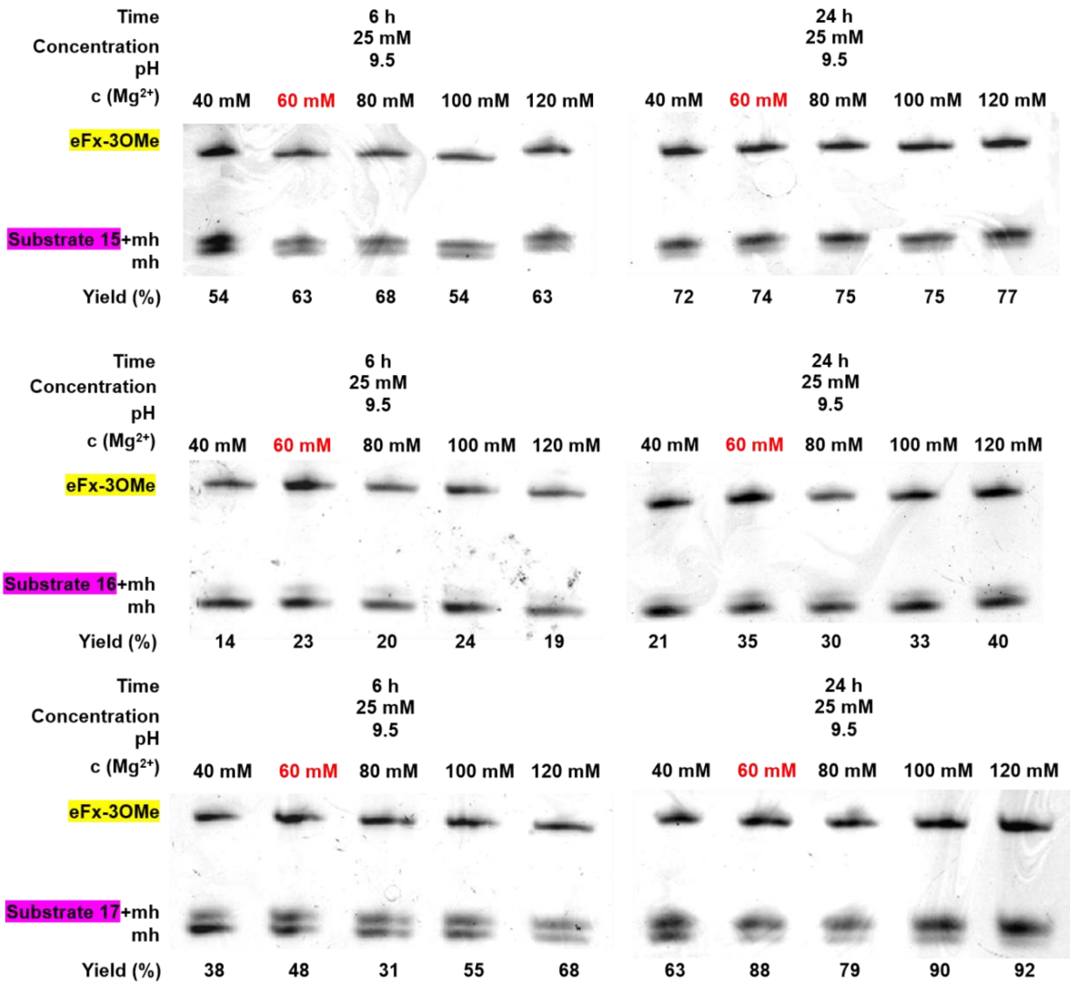


Supplementary Figure 9. The aminoacylation efficiency for eFx-3OMe and eFx with all acid-urea PAGE gel.

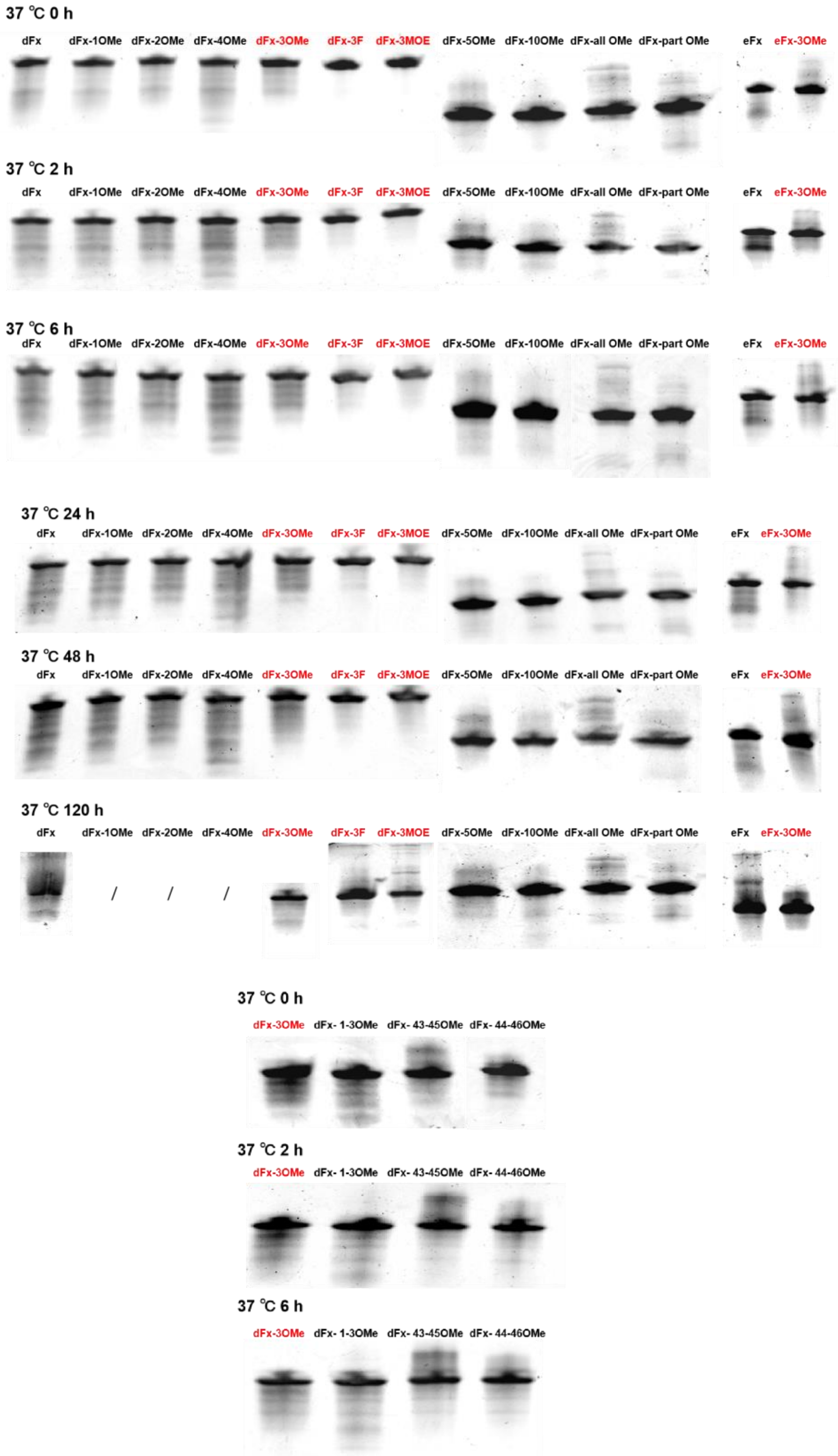
(a) The aminoacylation efficiency for eFx-3OMe and eFx with the acid-urea PAGE gel (triplicate measurements).
(b) The comparison of the aminoacylation efficiency for eFx-3OMe and eFx
E represents eFx-3OMe, **F** represents eFx.



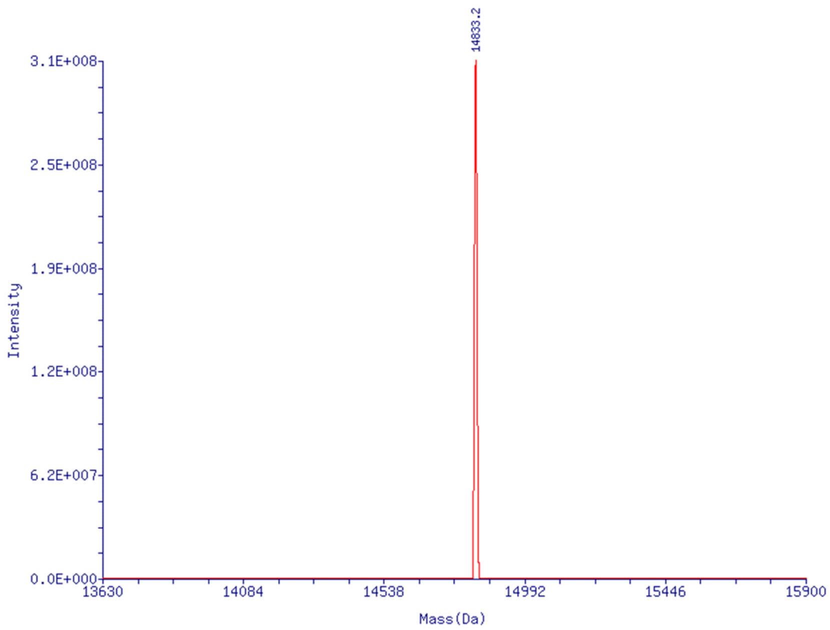
Supplementary Figure 10. The effect of Mg^{2+} concentration on the substrate with DBE groups on the aminoacylation efficiency of dFx-3OMe.



Supplementary Figure 11. The effect of Mg^{2+} concentration about the Substrate with CME groups on the aminoacylation efficiency of eFx-3OMe.

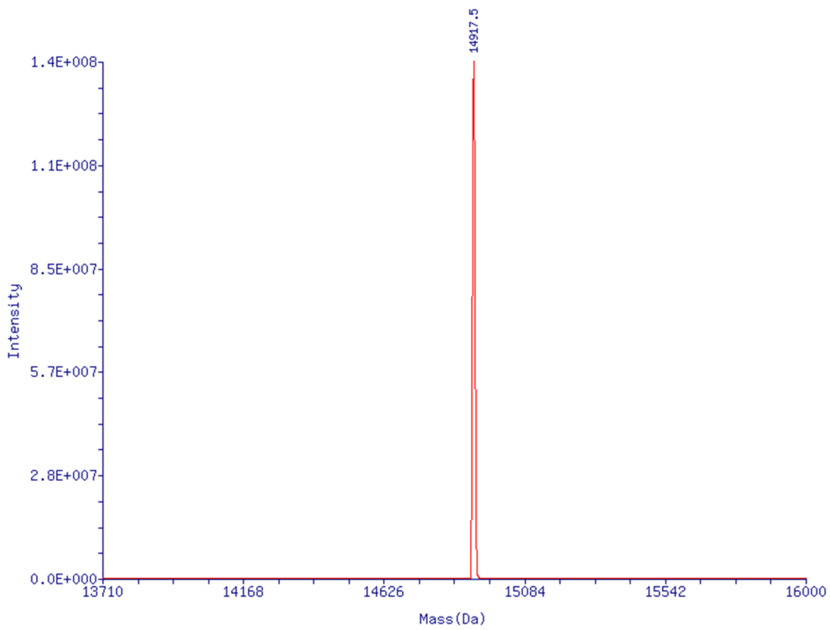


Supplementary Figure 12. The nuclease resistance of the wild-type and modified flexizymes.



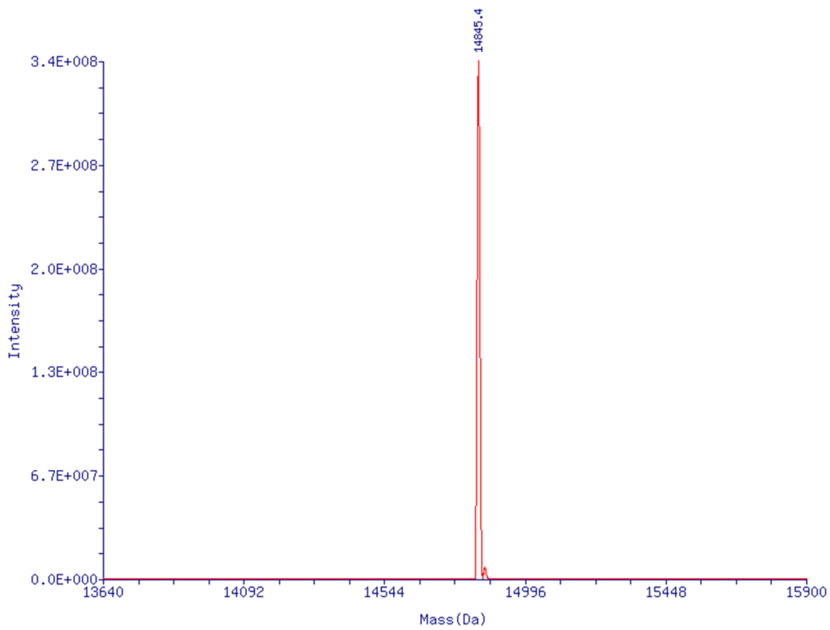
Supplementary Figure 13. The mass spectrum of the dFx.

Exact mass calcd. for dFx $[M+H]^+$ 14835.99, found 14833.2.



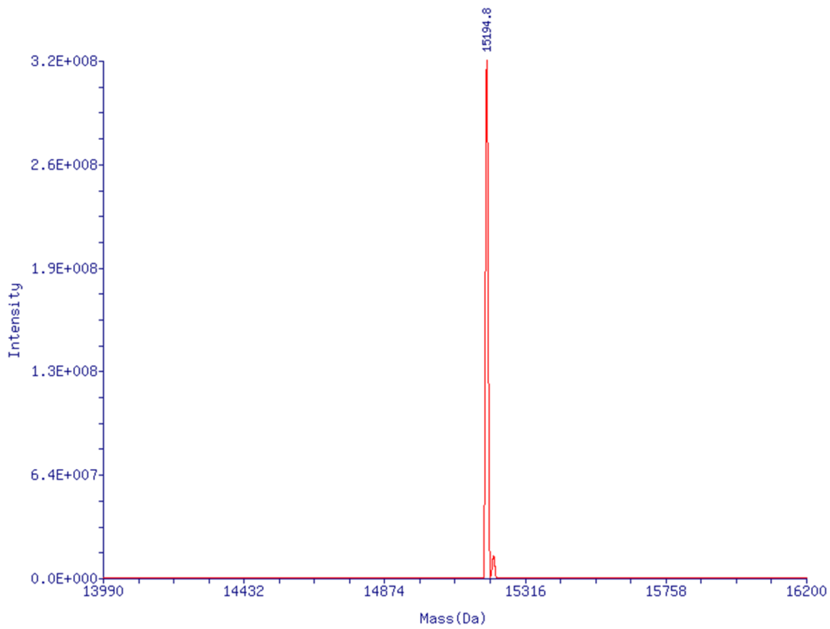
Supplementary Figure 14. The mass spectrum of the dFx-3OMe.

Exact mass calcd. for dFx-3OMe $[M+H]^+$ 14920.17, found 14917.5.



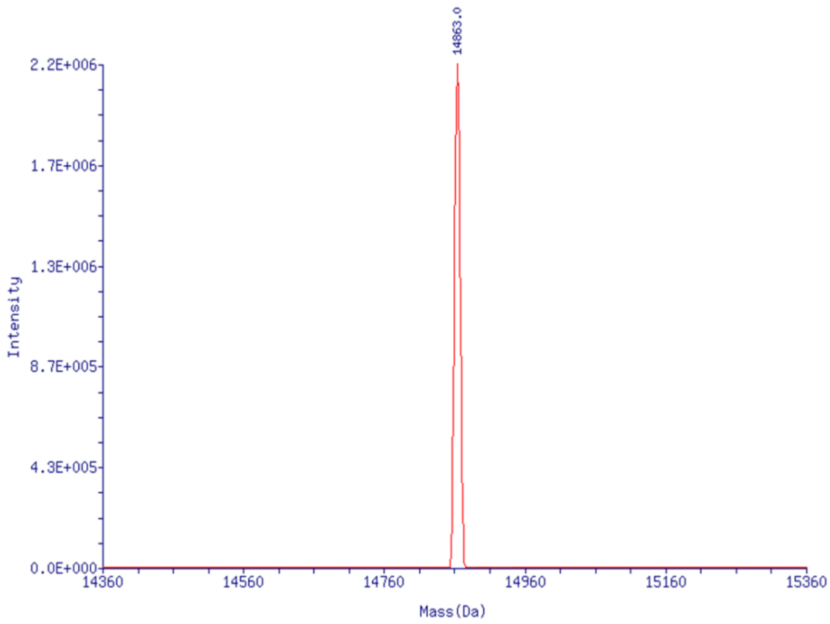
Supplementary Figure 15. The mass spectrum of the dFx-3F.

Exact mass calcd. for dFx-3F $[M+H]^+$ 14847.89, found 14845.4.



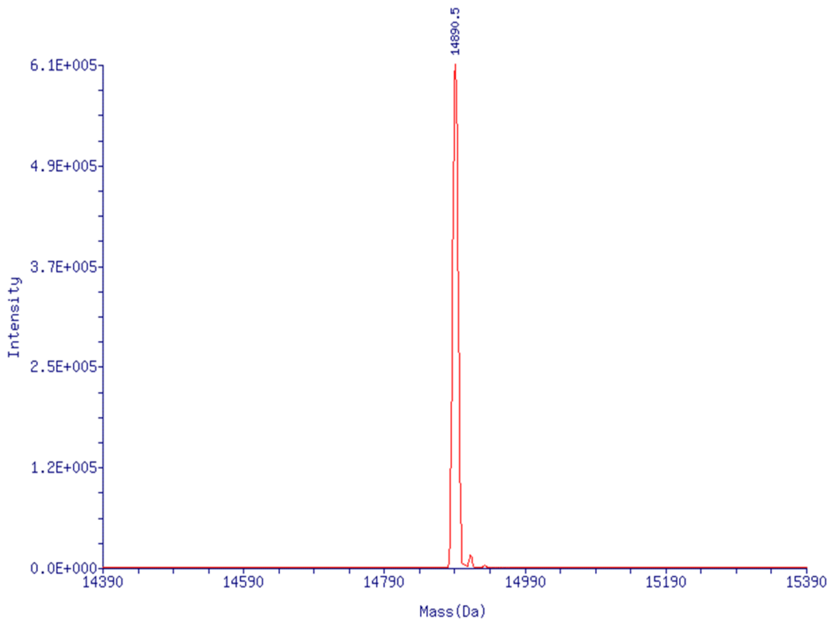
Supplementary Figure 16. The mass spectrum of the dFx-3MOE.

Exact mass calcd. for dFx-3MOE $[M+H]^+$ 15198.49, found 15194.8.



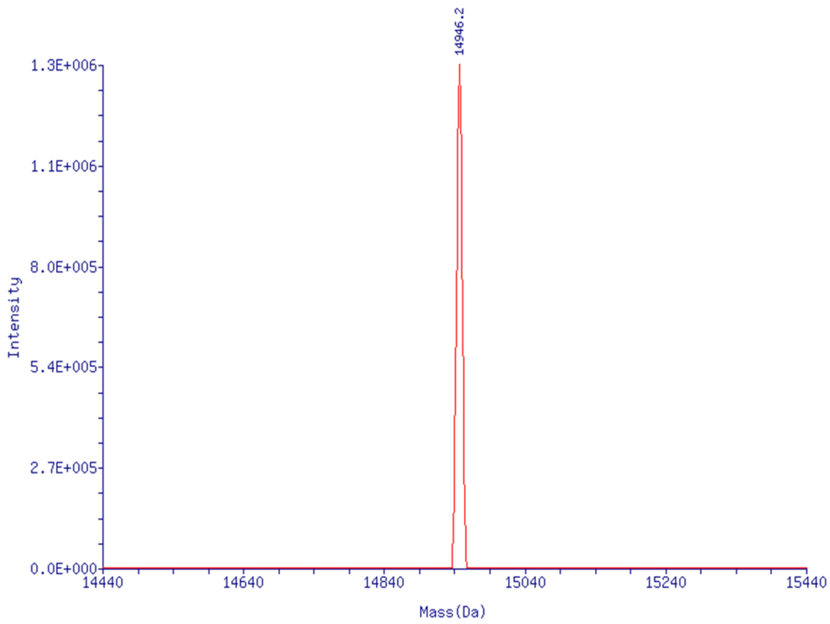
Supplementary Figure 17. The mass spectrum of the dFx-1OMe.

Exact mass calcd. for dFx-1OMe $[M+H]^+$ 14863.05, found 14863.0.



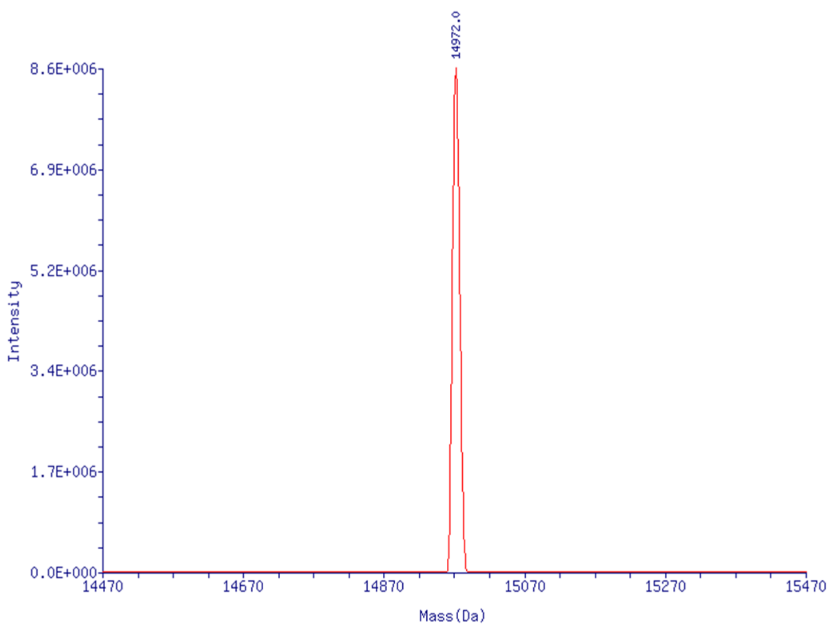
Supplementary Figure 18. The mass spectrum of the dFx-2OMe.

Exact mass calcd. for 1OMe $[M+H]^+$ 14891.11, found 14890.5.



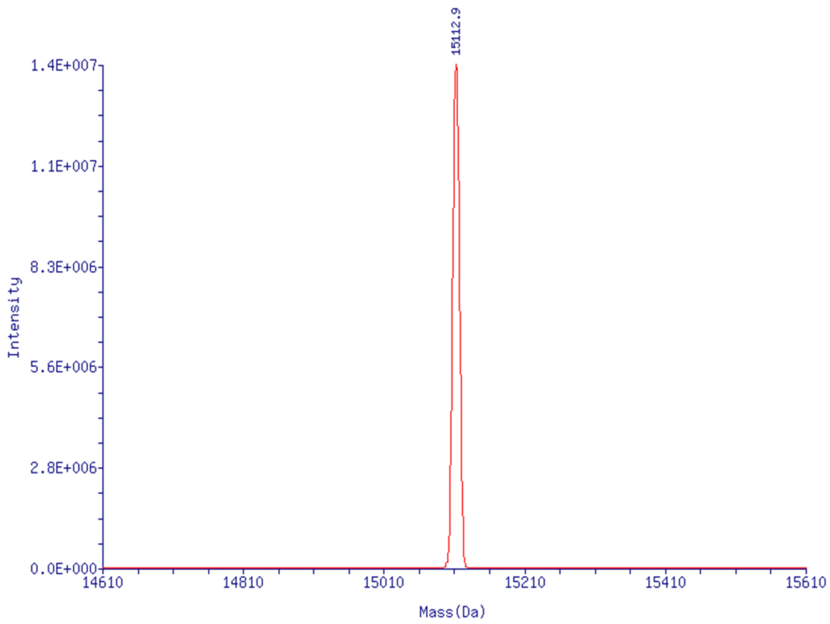
Supplementary Figure 19. The mass spectrum of the dFx-4OMe.

Exact mass calcd. for dFx-3F $[M+H]^+$ 14947.23, found 14946.2.



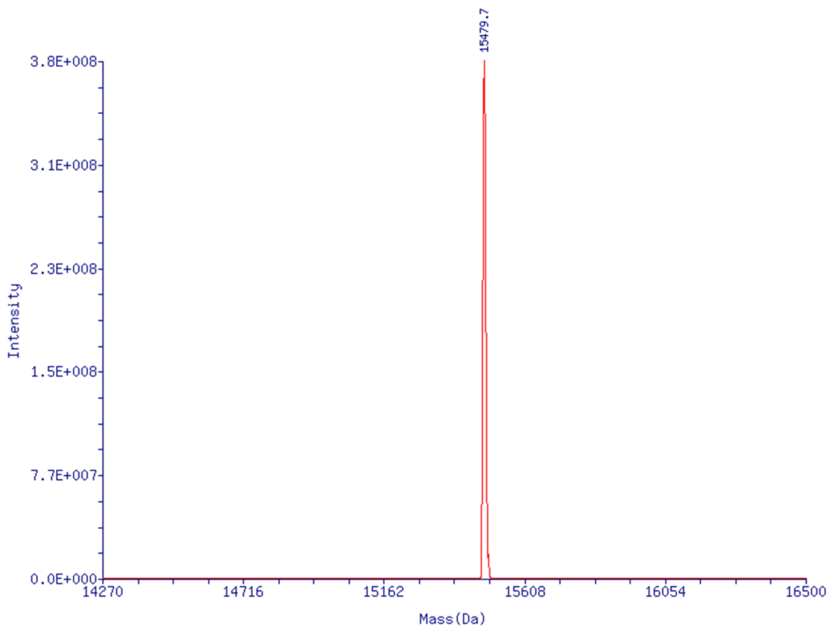
Supplementary Figure 20. The mass spectrum of the dFx-5OMe.

Exact mass calcd. for dFx-5OMe $[M+H]^+$ 14975.29, found 14972.0.



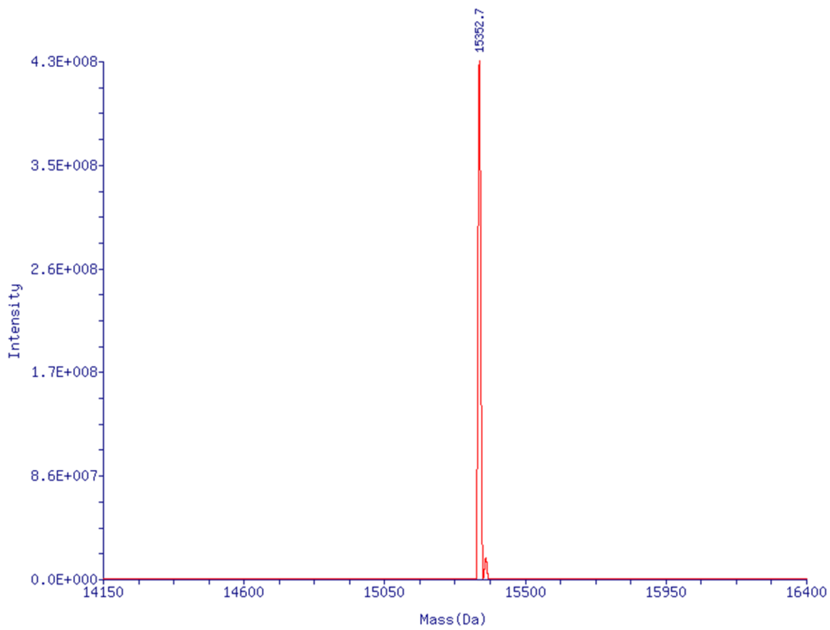
Supplementary Figure 21. The mass spectrum of the dFx-10OMe.

Exact mass calcd. for dFx-10OMe $[M+H]^+$ 15116.59, found 15112.9.



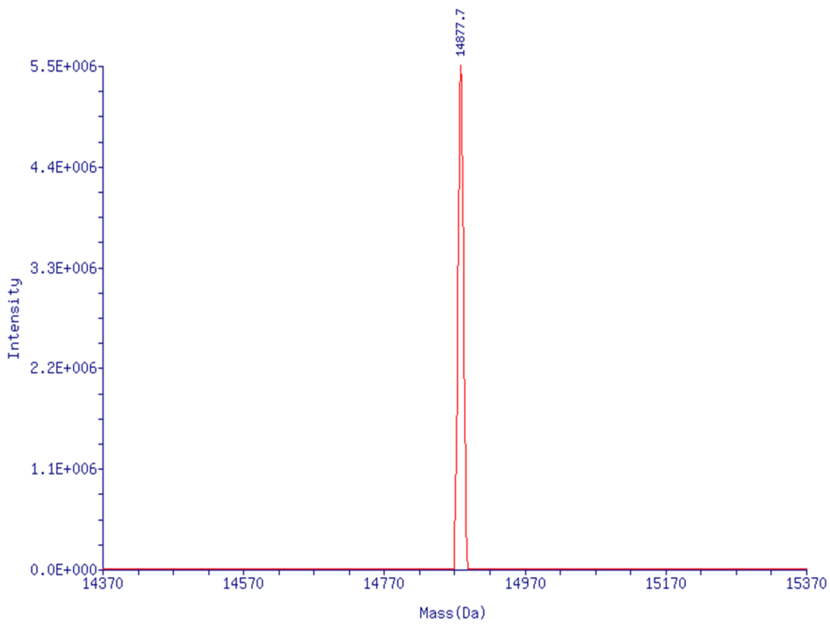
Supplementary Figure 22. The mass spectrum of the dFx-all OMe.

Exact mass calcd. for dFx-all OMe $[M+H]^+$ 15481.37, found 15479.7.



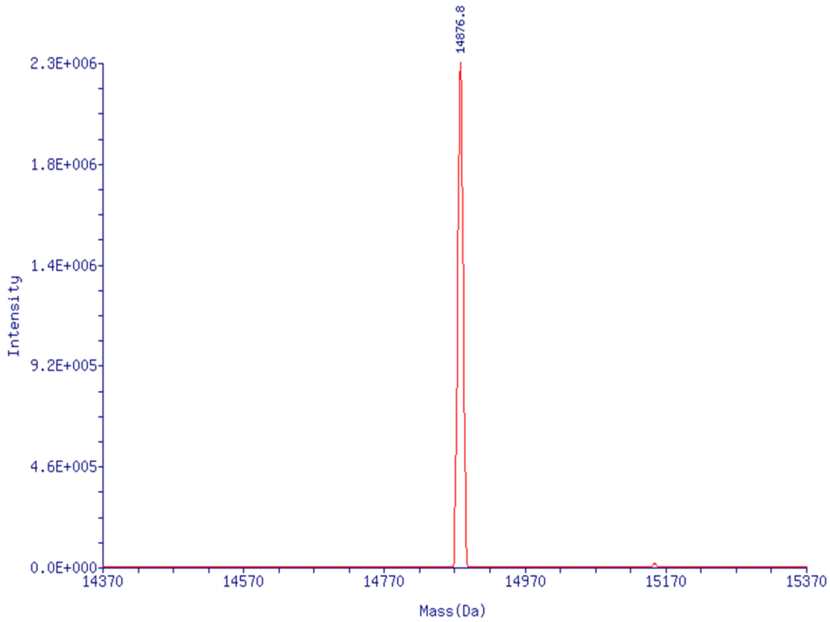
Supplementary Figure 23. The mass spectrum of the dFx-part OMe.

Exact mass calcd. for dFx-part OMe $[M+H]^+$ 15355.10, found 15352.7



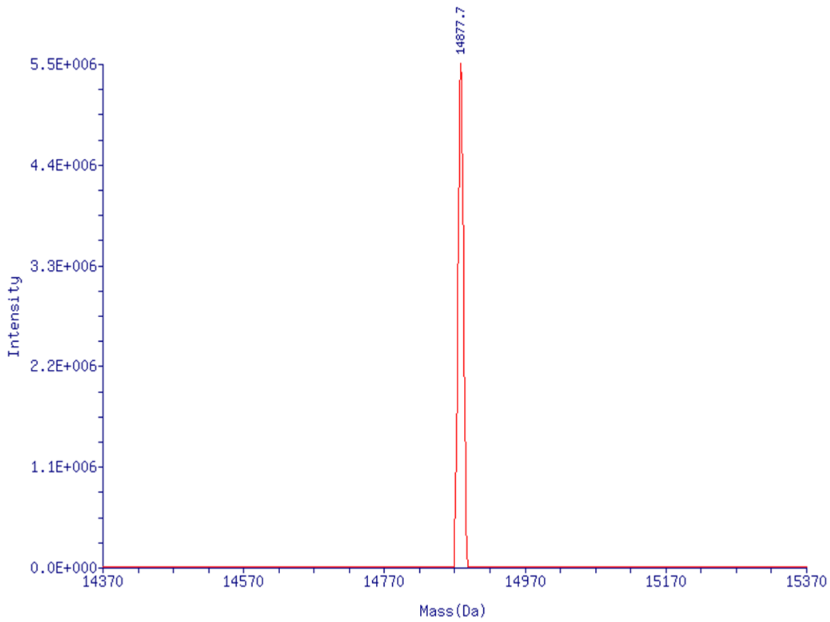
Supplementary Figure 24. The mass spectrum of the dFx- 1-3OMe.

Exact mass calcd. for dFx- 1-3 OMe $[M+H]^+$ 14877.08, found 14877.7.



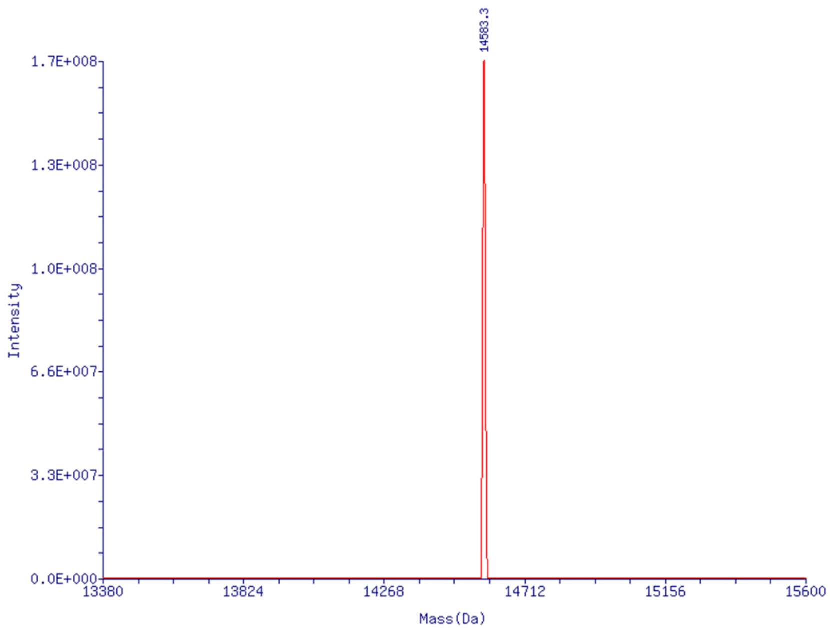
Supplementary Figure 25. The mass spectrum of the dFx- 43-45OMe.

Exact mass calcd. for dFx- 43-45 OMe $[M+H]^+$ 14877.08, found 14876.8.



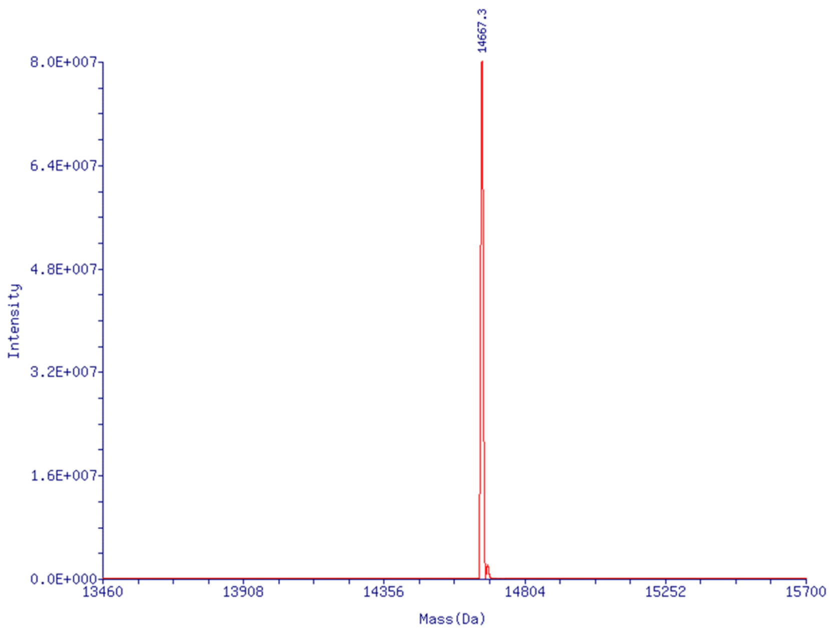
Supplementary Figure 26. The mass spectrum of the dFx- 44-46OMe.

Exact mass calcd. for dFx- 44-46 OMe $[M+H]^+$ 14877.08, found 14877.7.



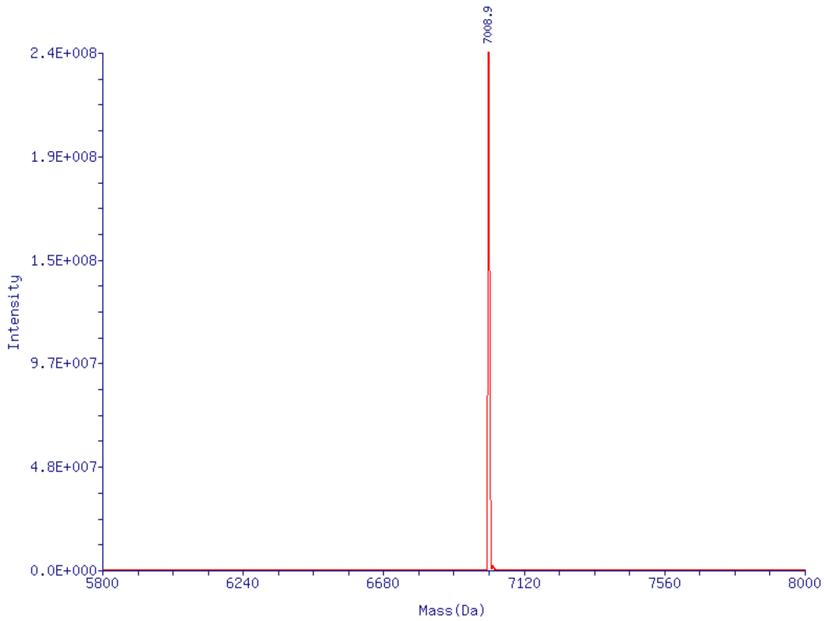
Supplementary Figure 27. The mass spectrum of the eFx.

Exact mass calcd. for eFx $[M+H]^+$ 14585.85, found 14583.3.



Supplementary Figure 28. The mass spectrum of the eFx-3OMe.

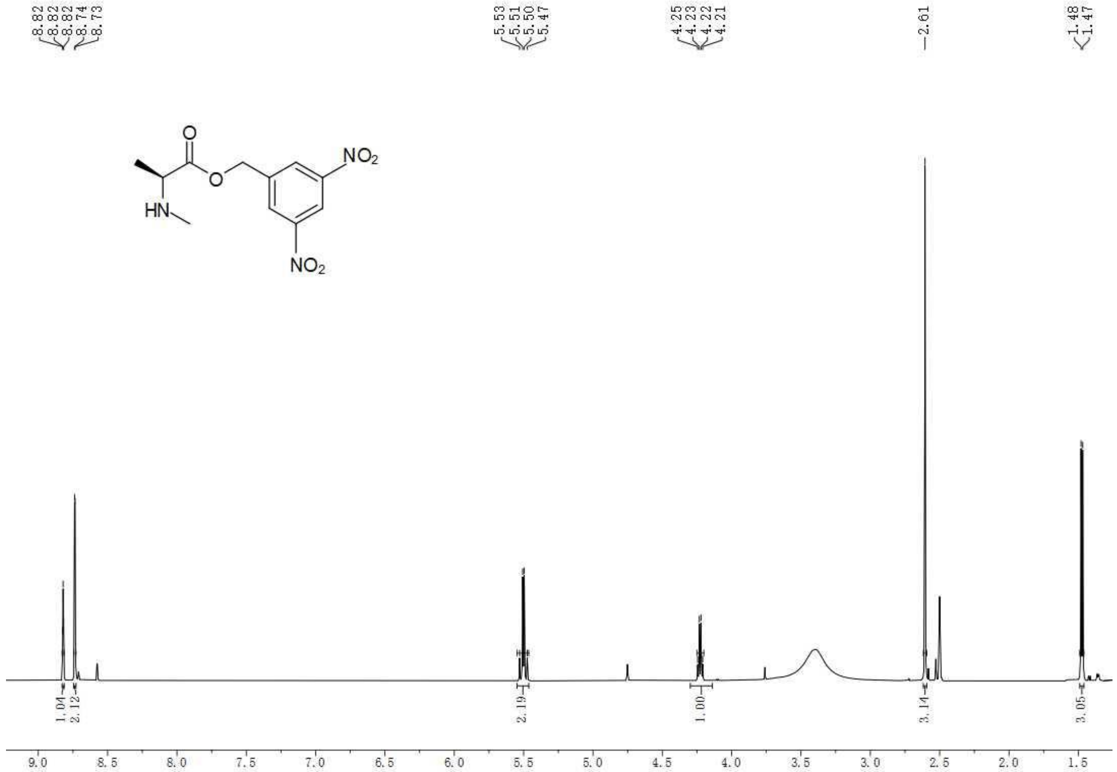
Exact mass calcd. for eFx -3OMe$[M+H]^+$ 14670.03, found 14667.3.



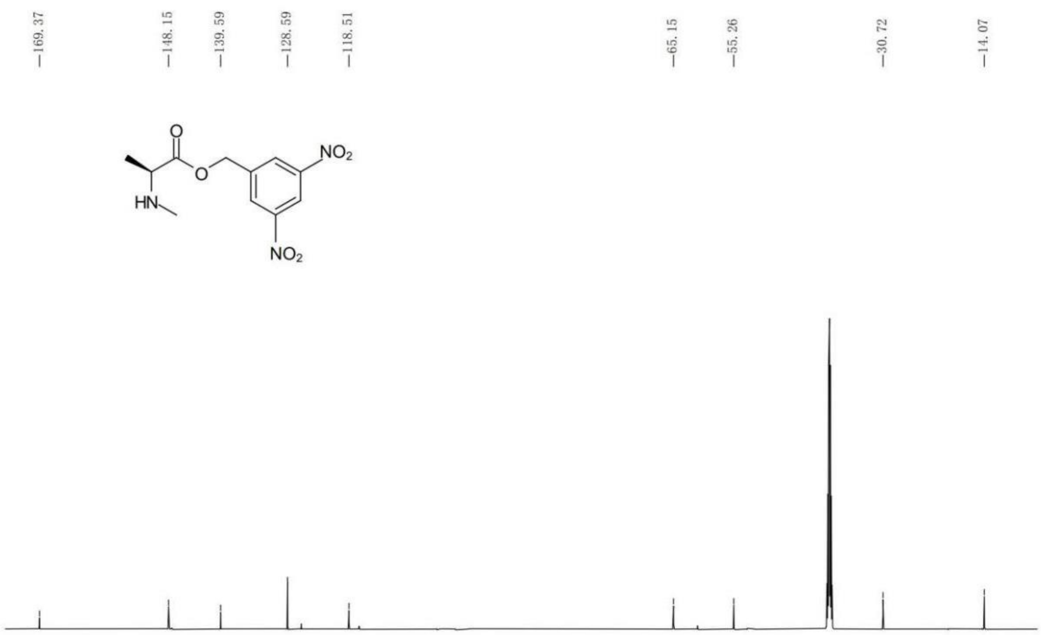
Supplementary Figure 29. The mass spectrum of the microhelix.

Exact mass calcd. for microhelix$[M+H]^+$ 7009.25, found 7008.9.

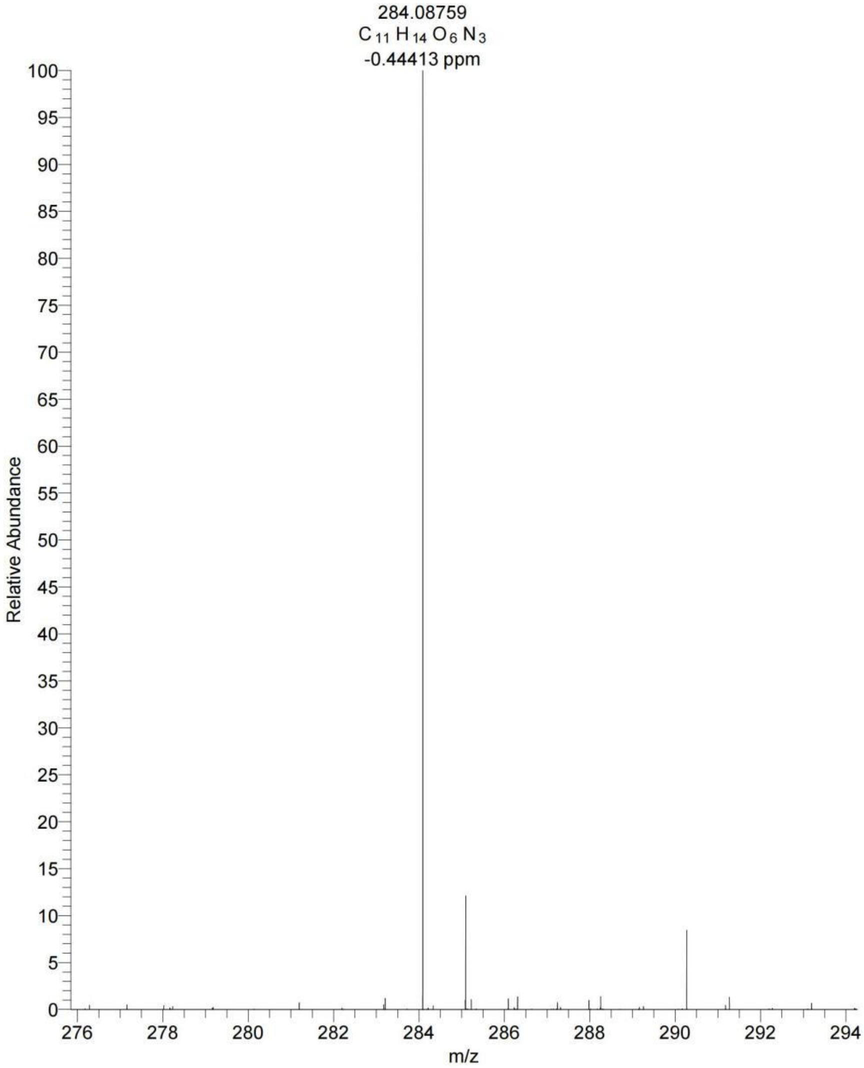
Supplementary Figure 30. ^{1}H NMR (600 MHz, DMSO-d_6) of 1.



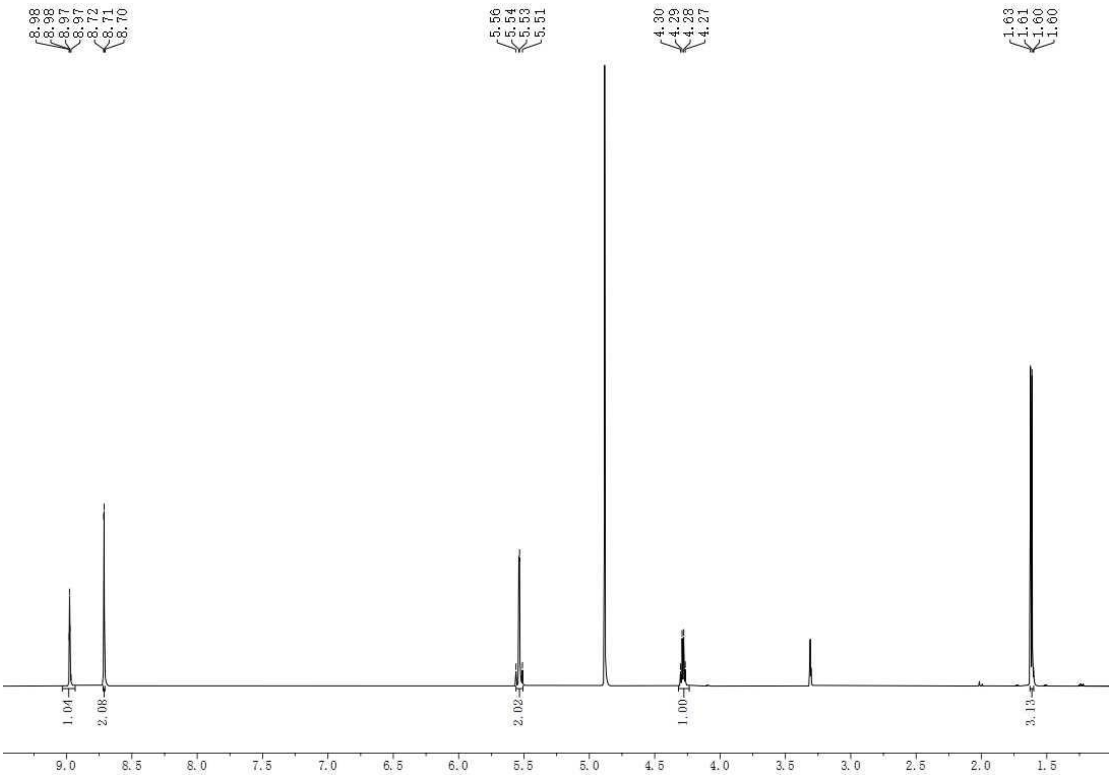
Supplementary Figure 31. ^{13}C NMR (150 MHz, DMSO-d_6) of 1.



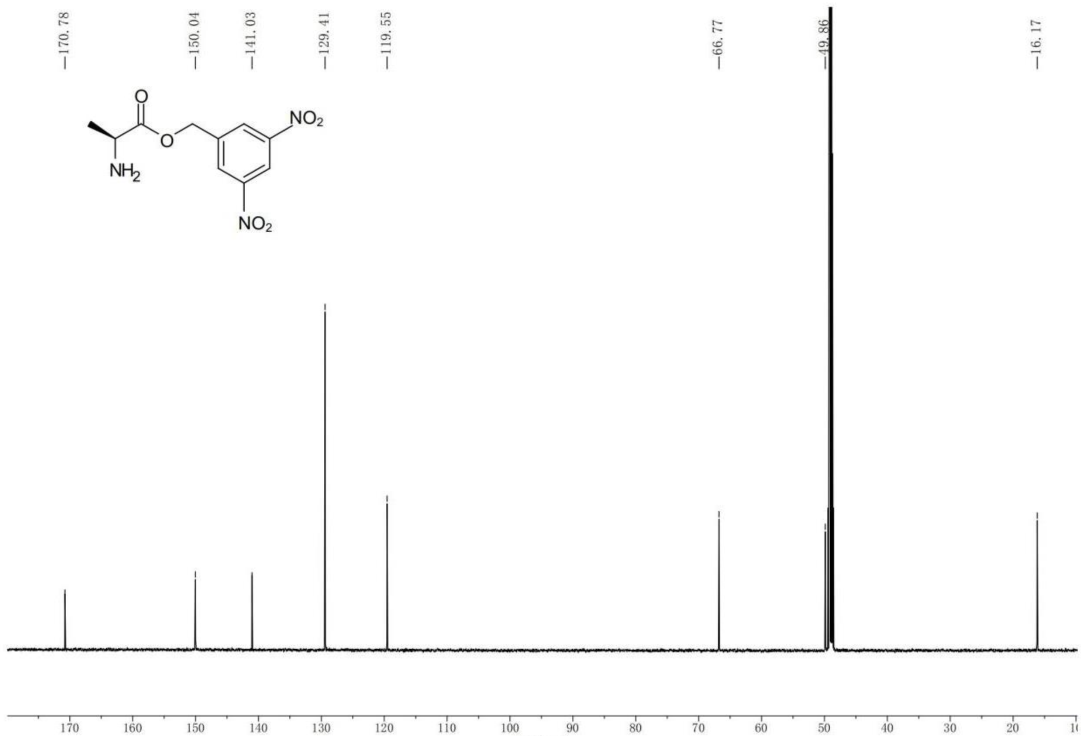
Supplementary Figure 32. The mass spectrum of 1.



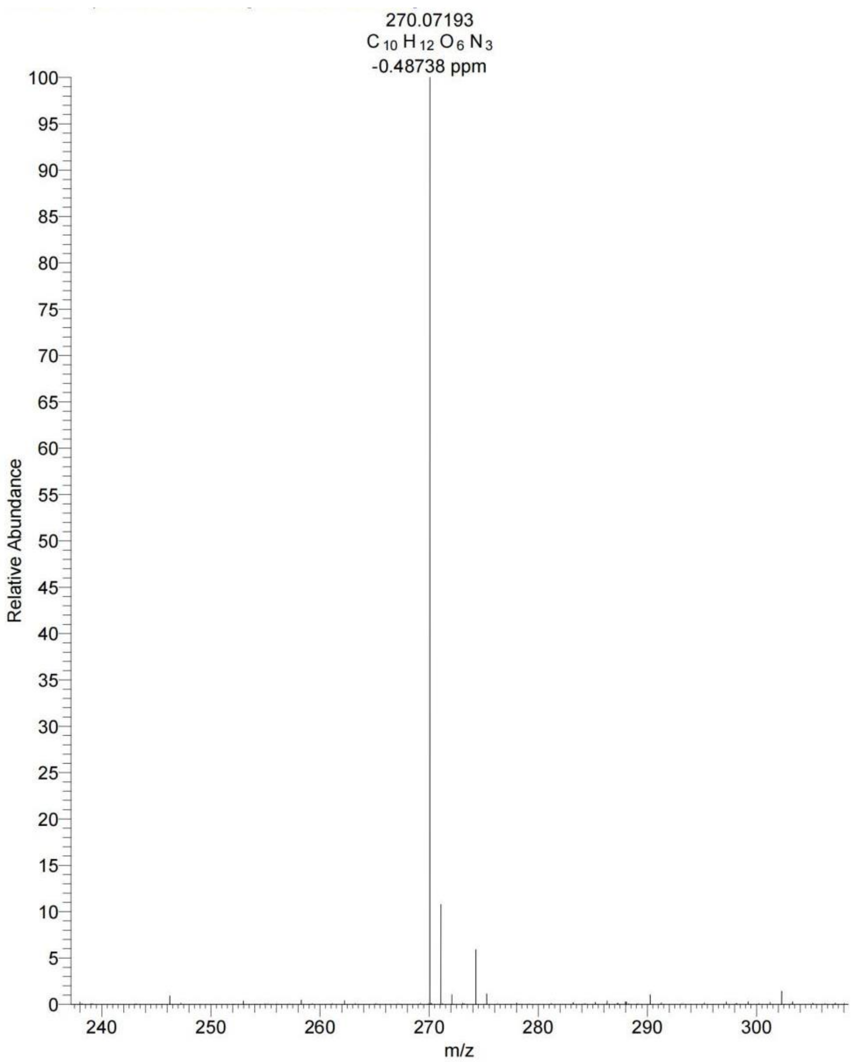
Supplementary Figure 33. ^{1}H NMR (600 MHz, MeOD) of 2.



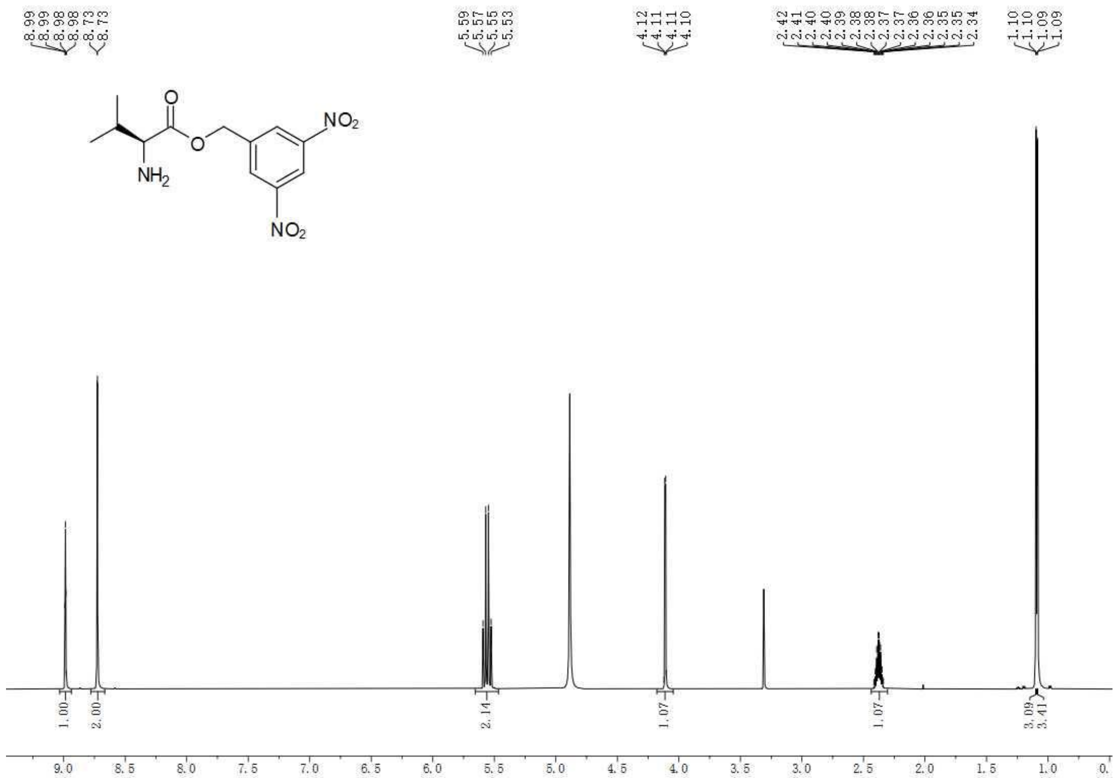
Supplementary Figure 34. ^{13}C NMR (150 MHz, MeOD) of 2.



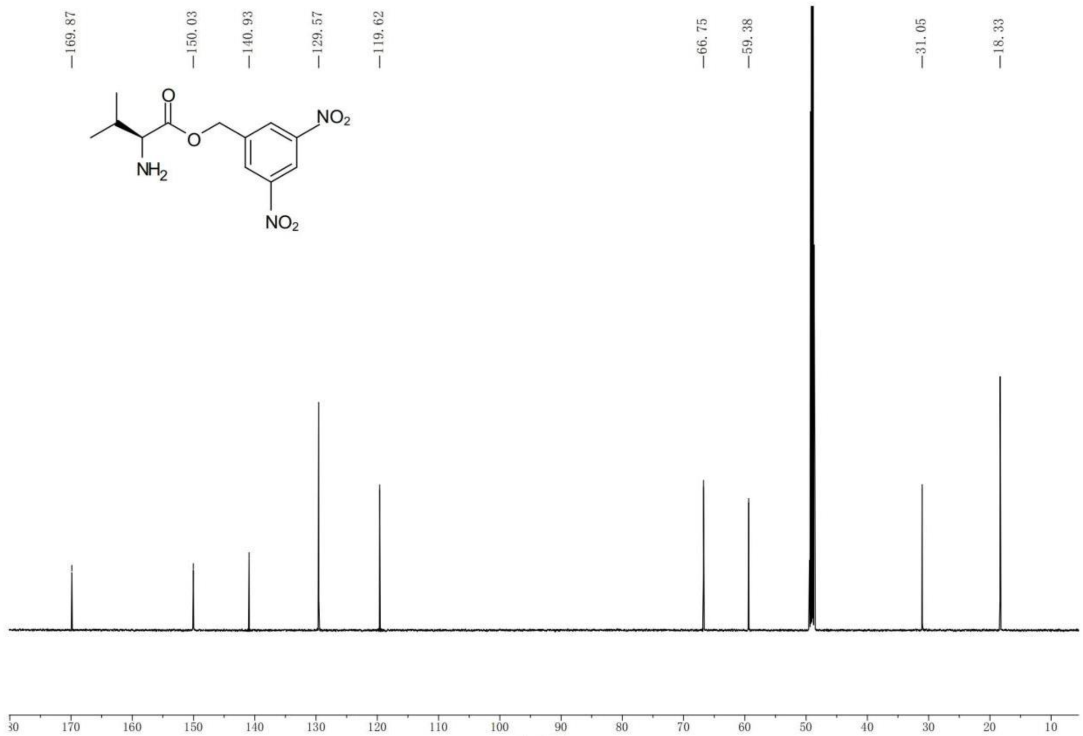
Supplementary Figure 35. The mass spectrum of 2.



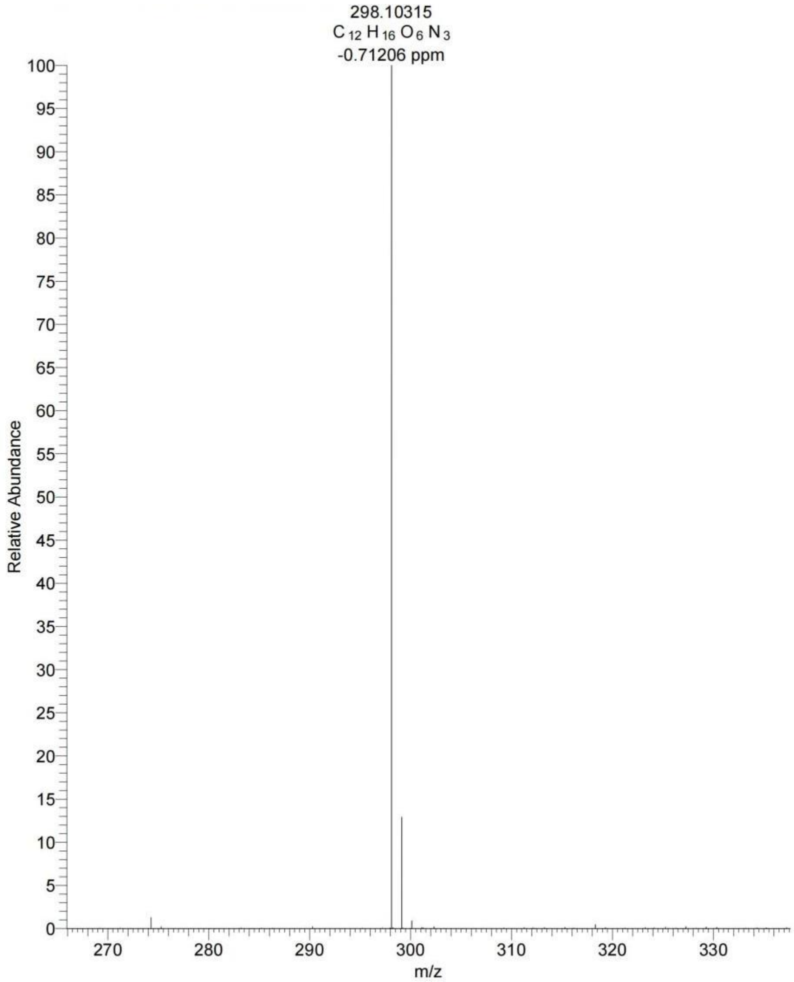
Supplementary Figure 36. ^{1}H NMR (600 MHz, MeOD) of 3.



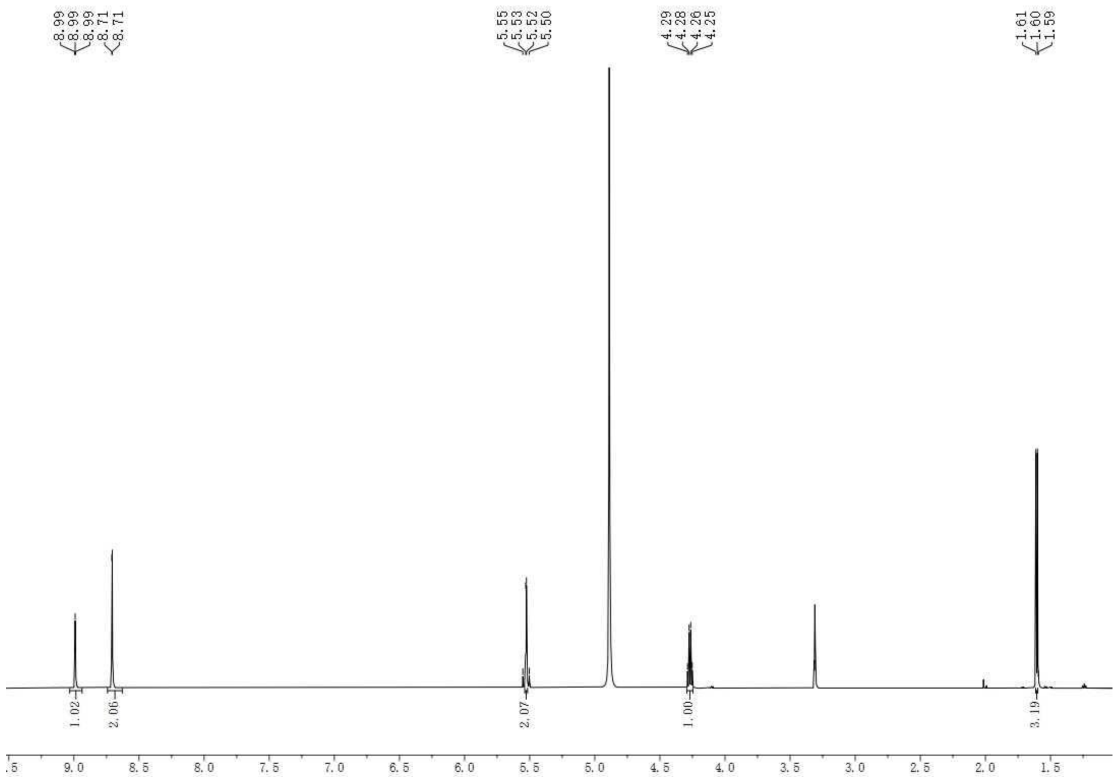
Supplementary Figure 37. ^{13}C NMR (150 MHz, MeOD) of 3.



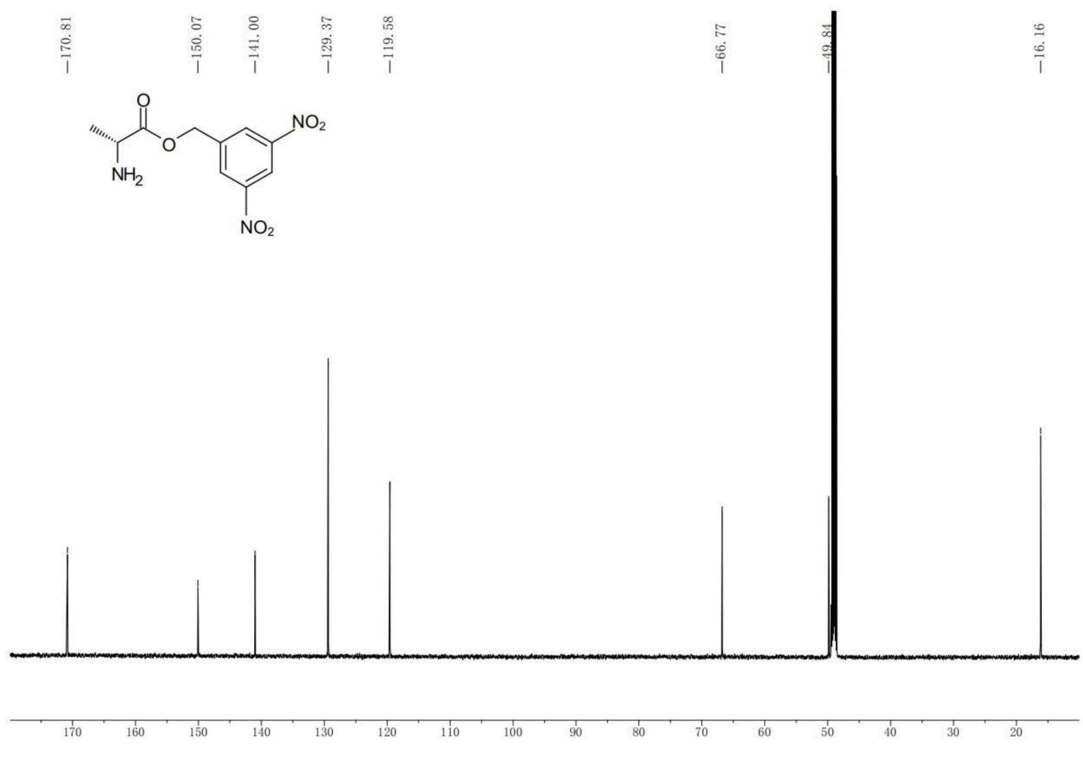
Supplementary Figure 38. The mass spectrum of 3.



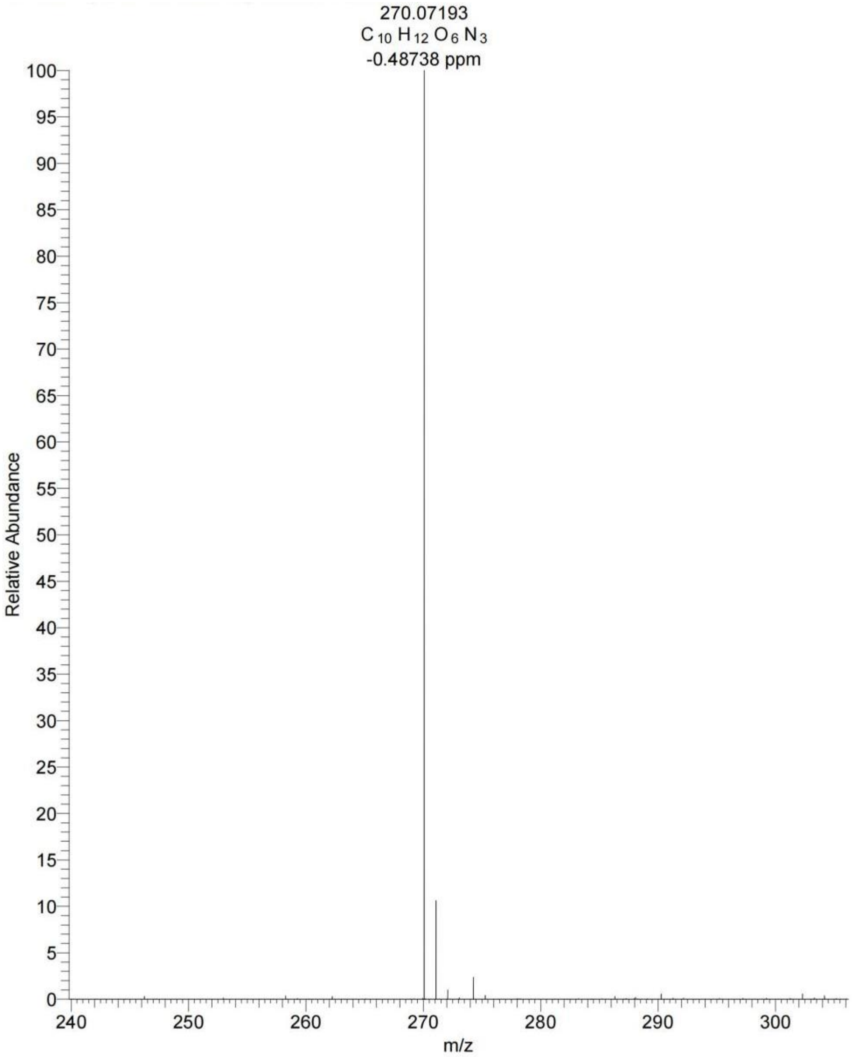
Supplementary Figure 39. ^{1}H NMR (600 MHz, MeOD) of 4.



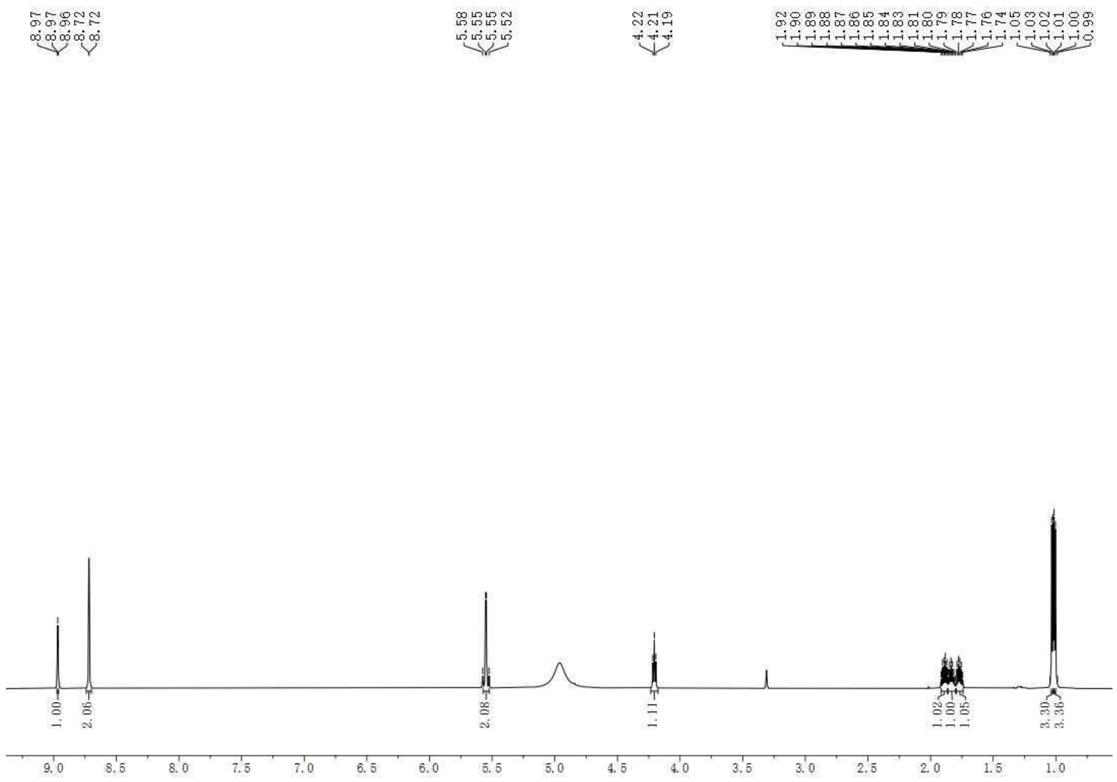
Supplementary Figure 40. ^{13}C NMR (150 MHz, MeOD) of 4.



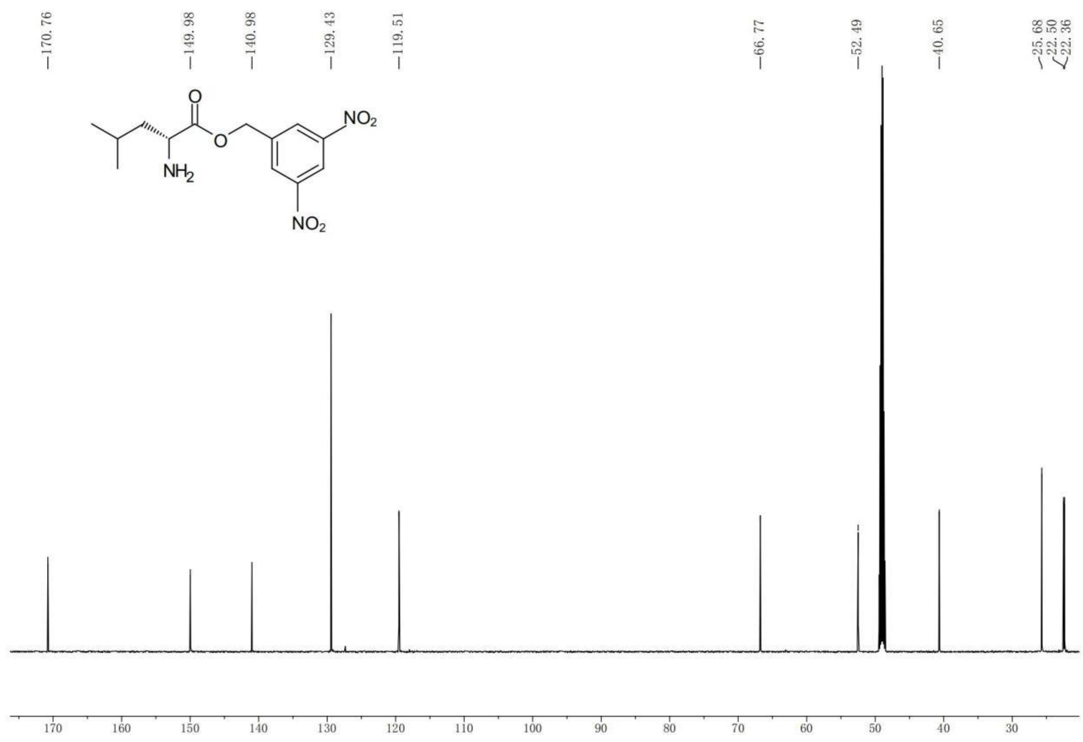
Supplementary Figure 41. The mass spectrum of 4.



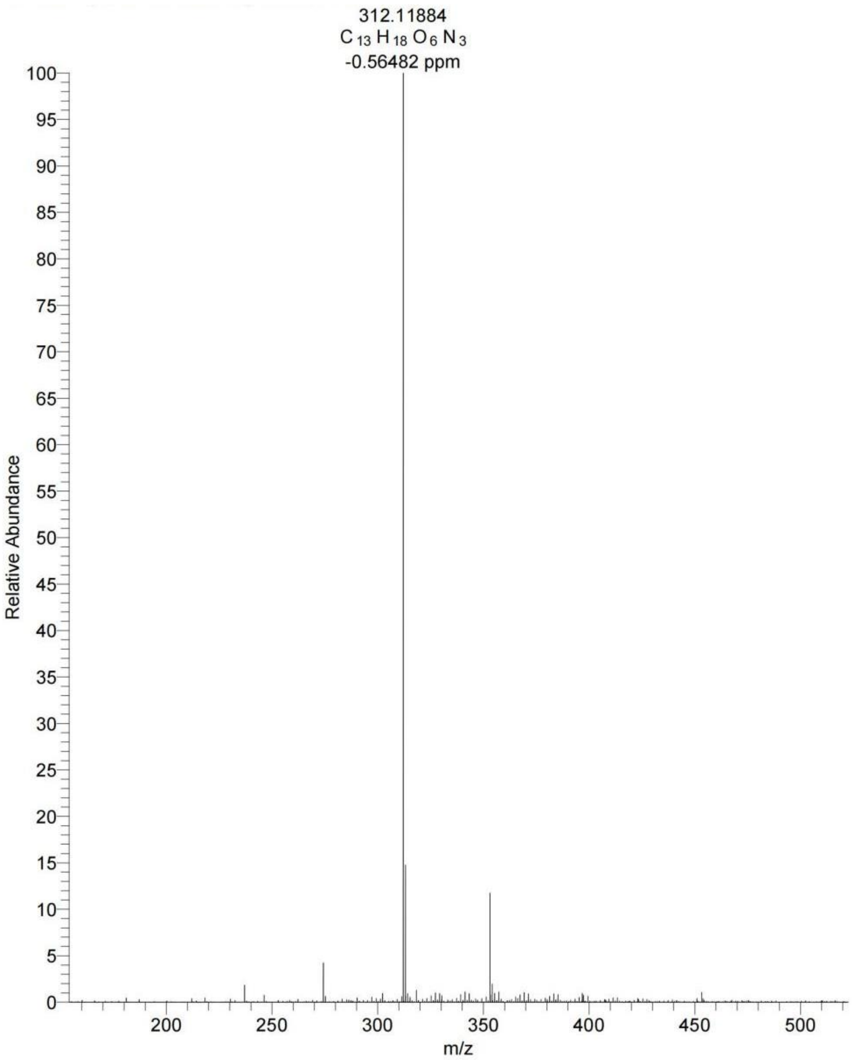
Supplementary Figure 42. [1]H NMR (600 MHz, MeOD) of 5.



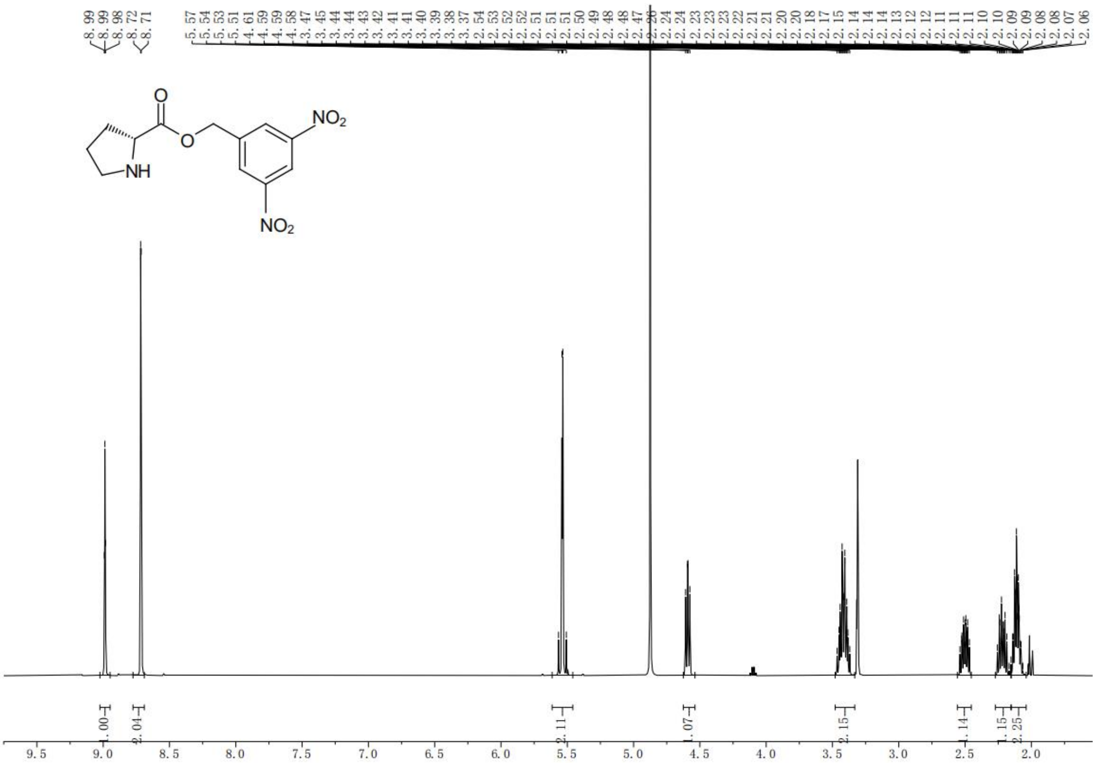
Supplementary Figure 43. [13]C NMR (150 MHz, MeOD) of 5.



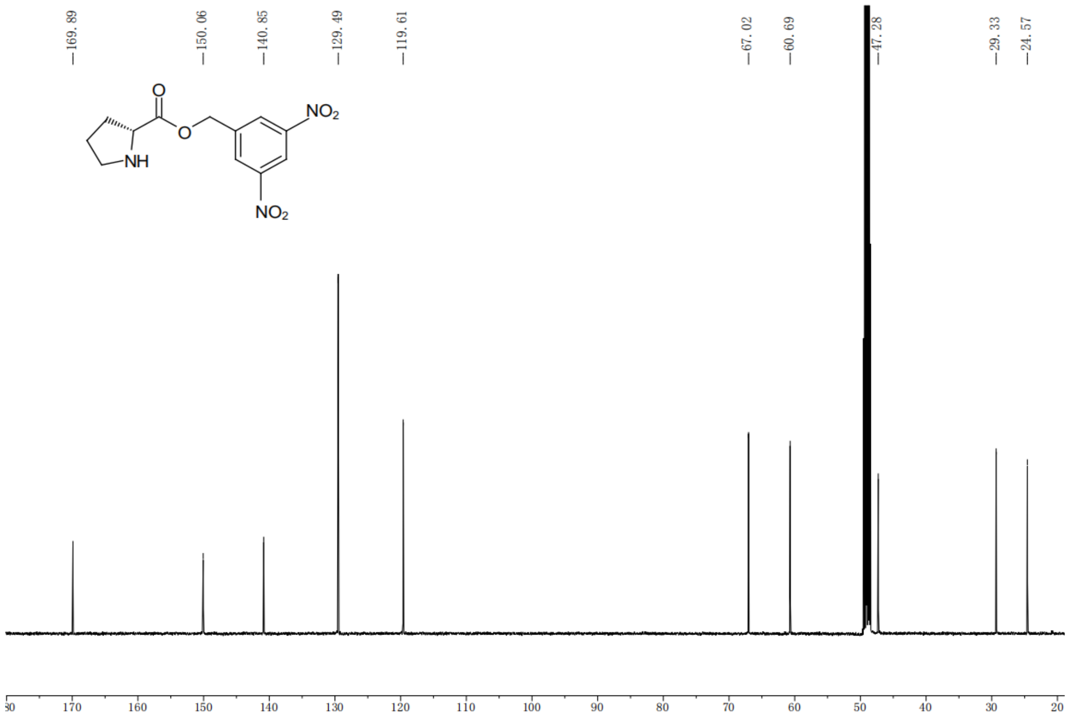
Supplementary Figure 44. The mass spectrum of 5.



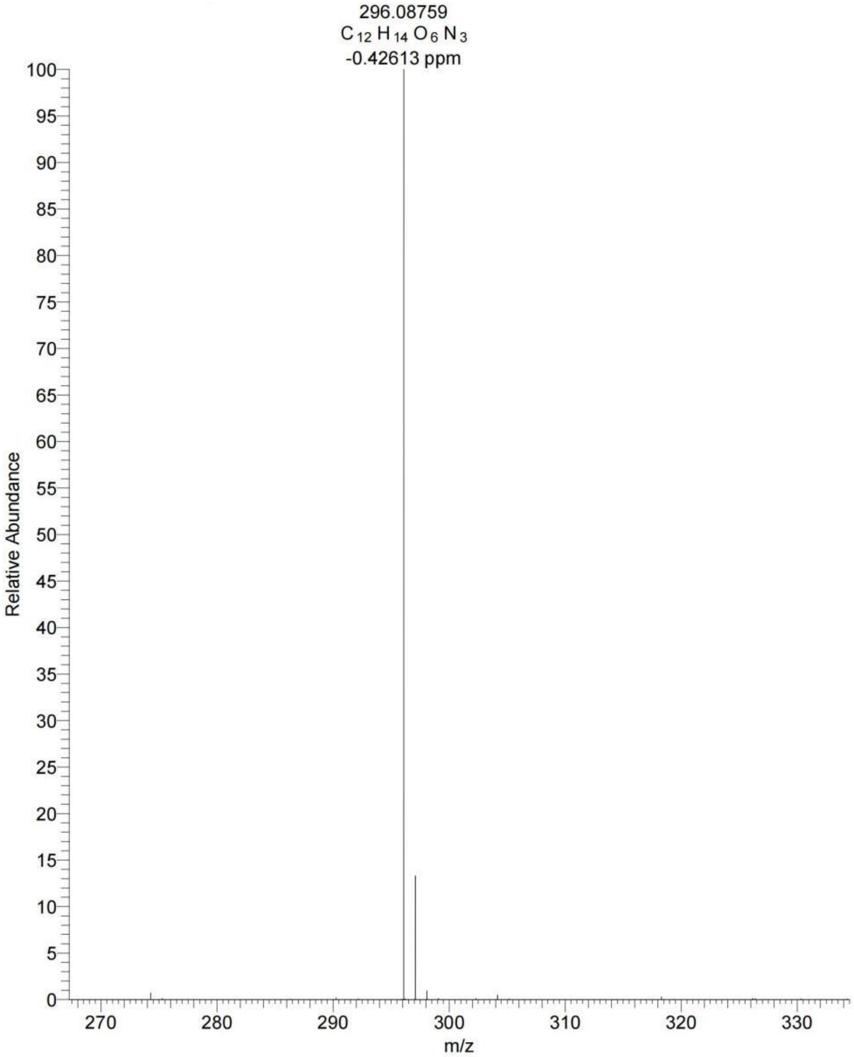
Supplementary Figure 45. ^{1}H NMR (500 MHz, MeOD) of 6.



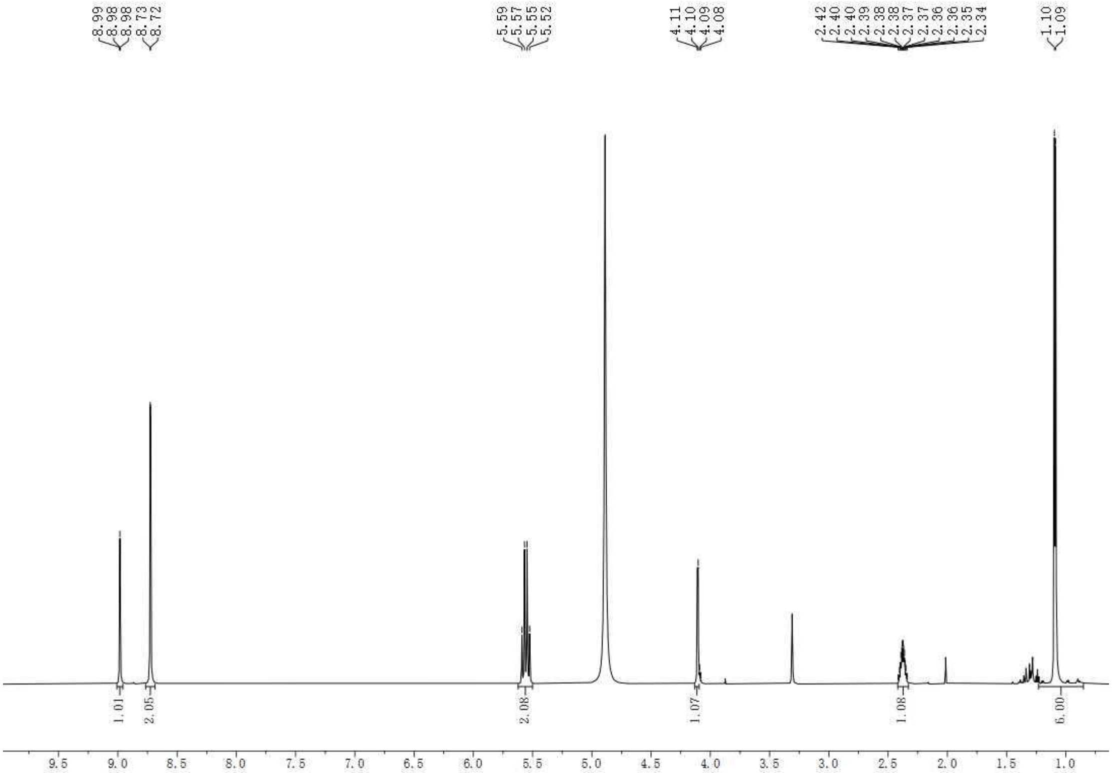
Supplementary Figure 46. ^{13}C NMR (125 MHz, MeOD) of 6.



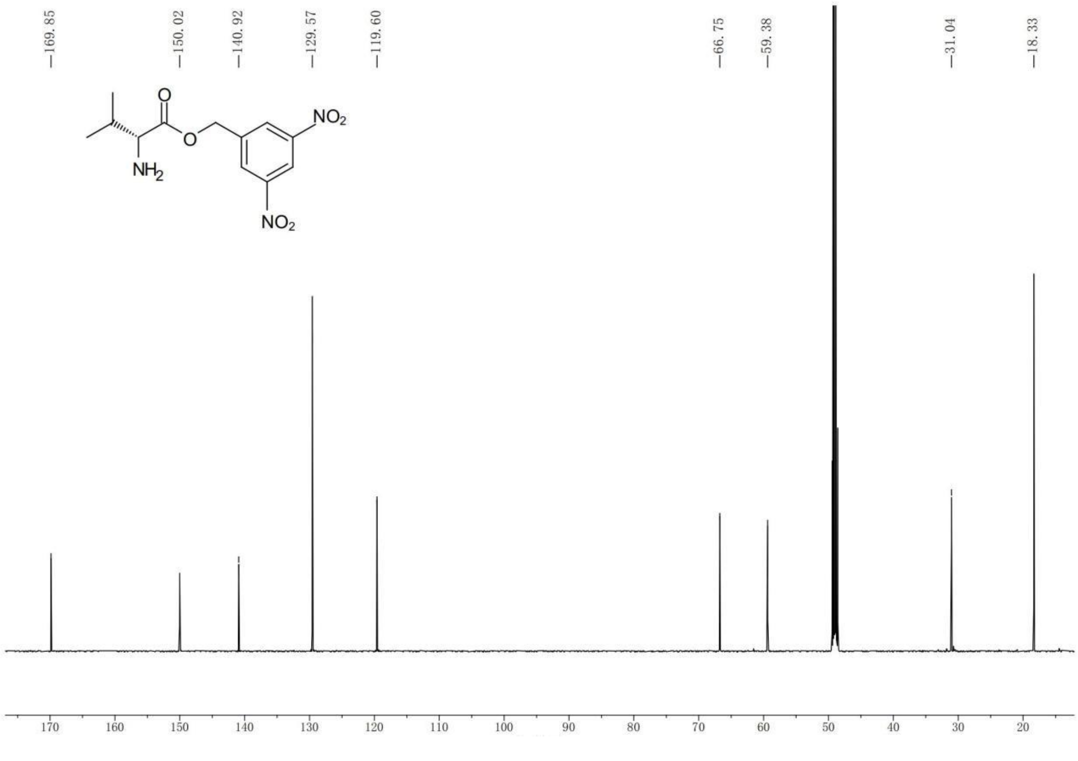
Supplementary Figure 47. The mass spectrum of 6.



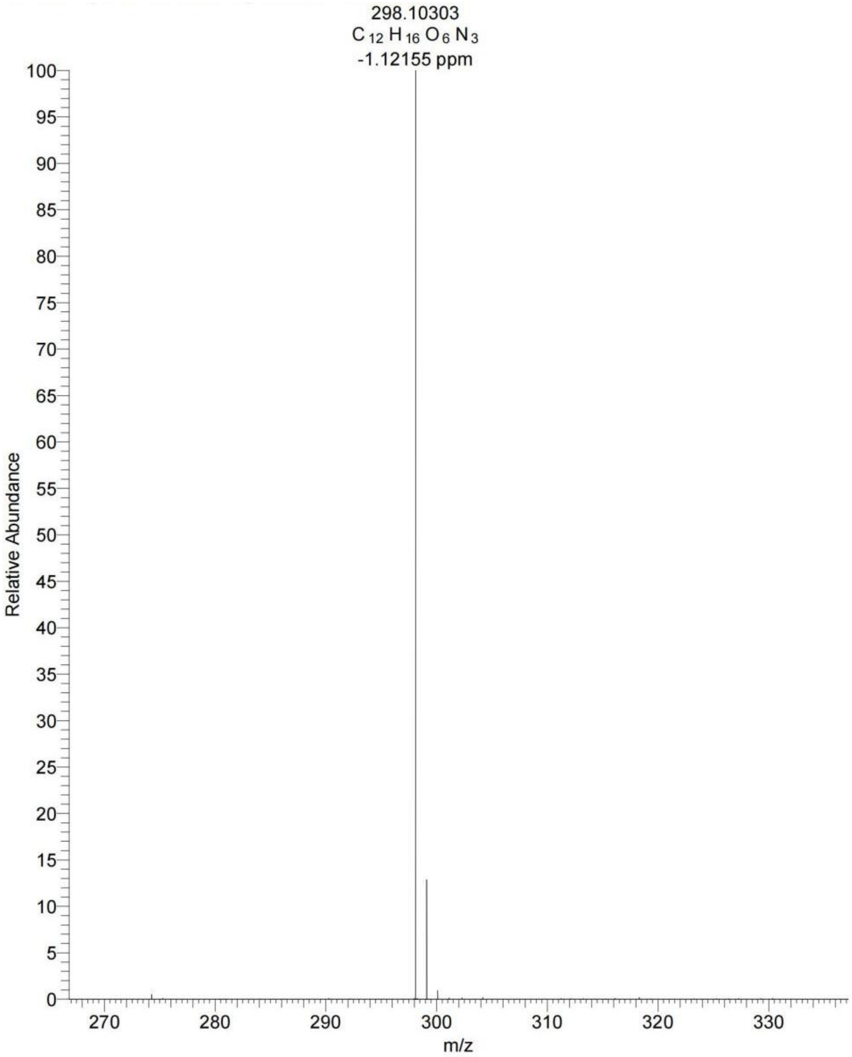
Supplementary Figure 48. ^{1}H NMR (500 MHz, MeOD) of 7.



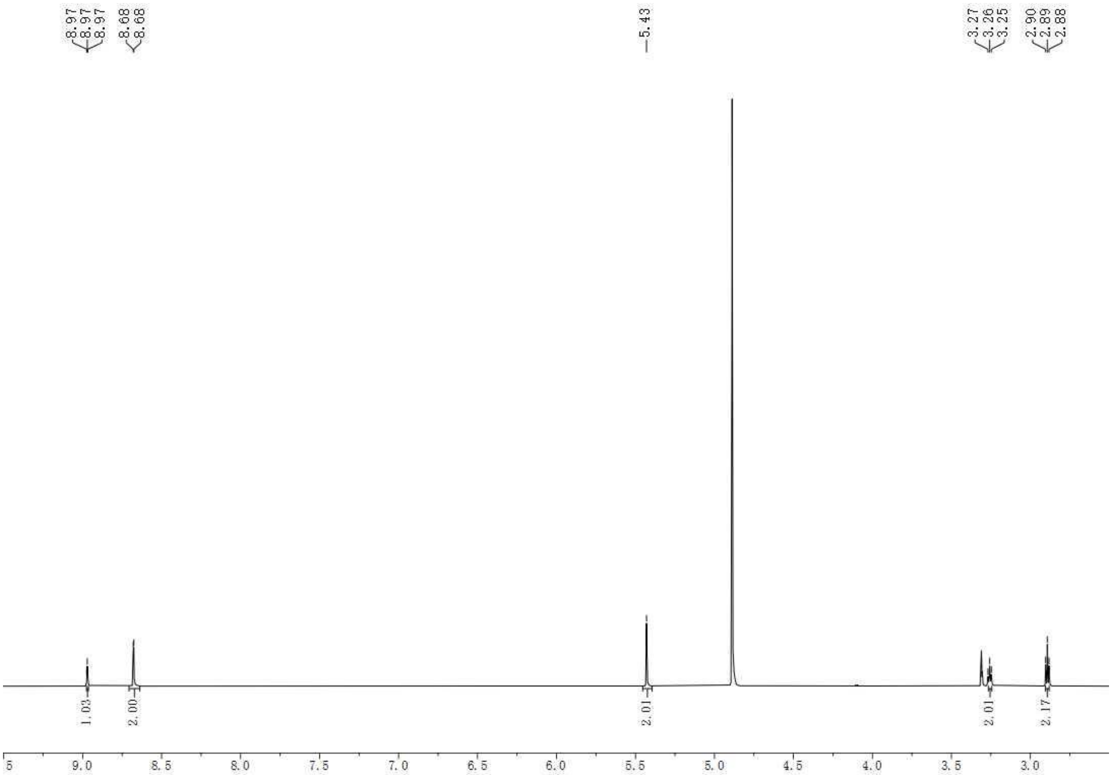
Supplementary Figure 49. ^{13}C NMR (150 MHz, MeOD) of 7.



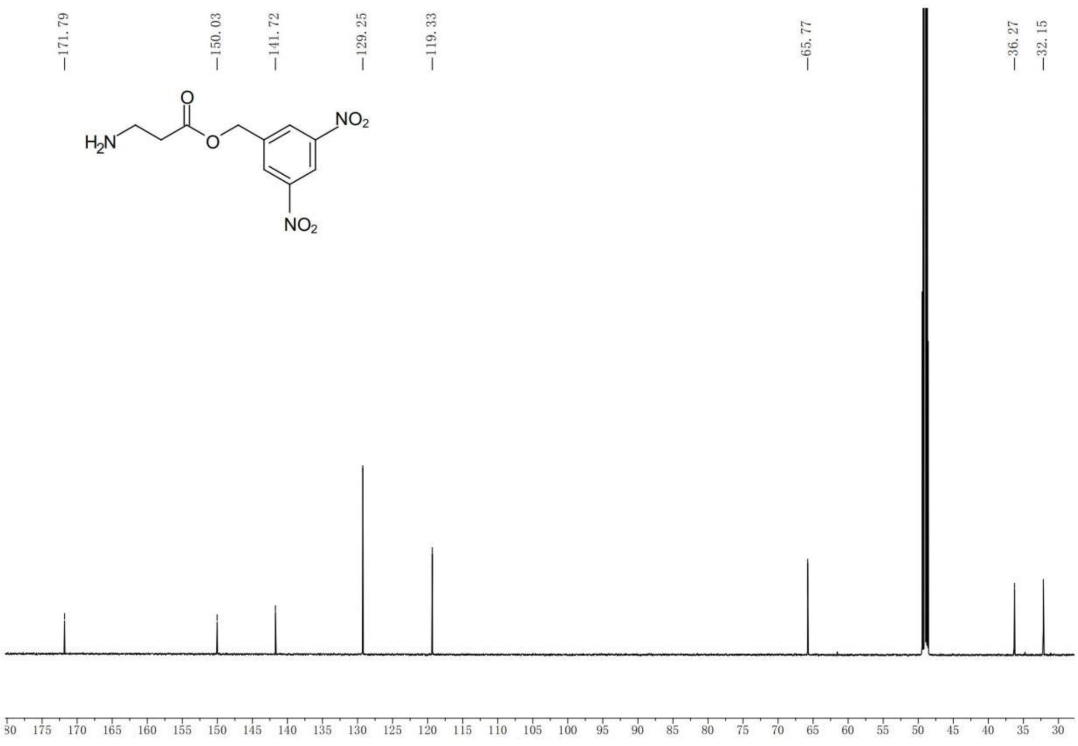
Supplementary Figure 50. The mass spectrum of 7.



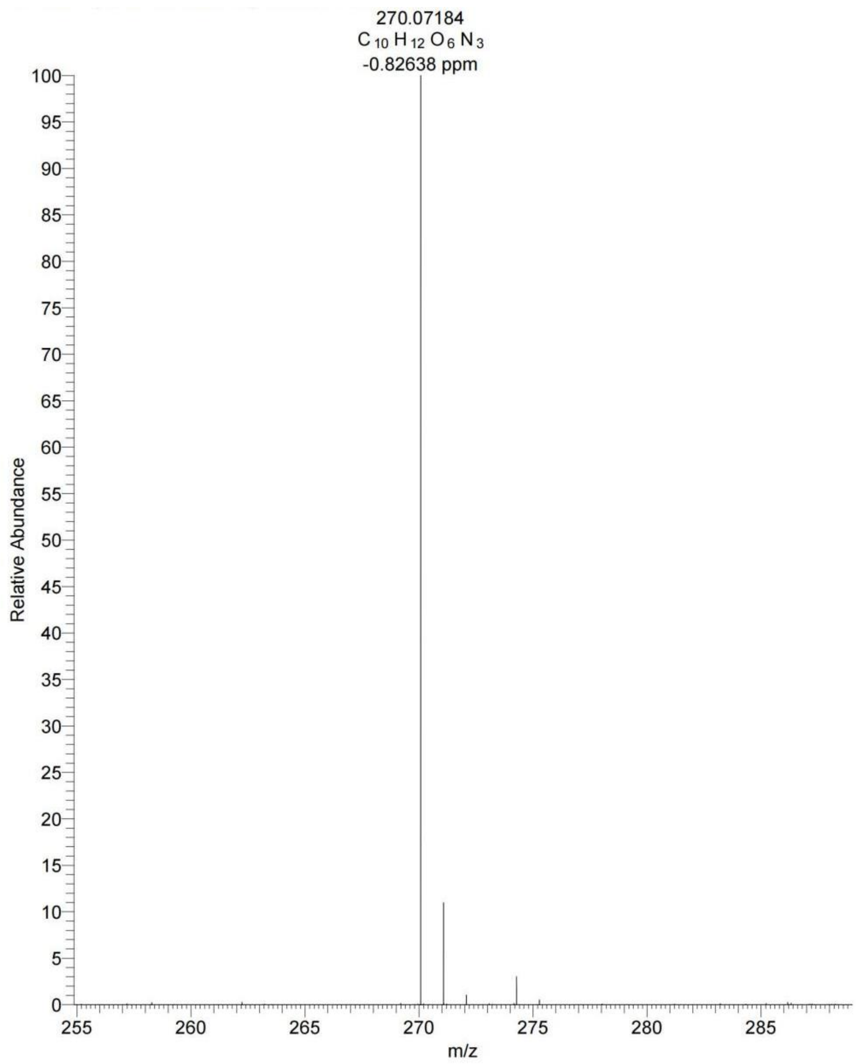
Supplementary Figure 51. ^{1}H NMR (600 MHz, MeOD) of 8.



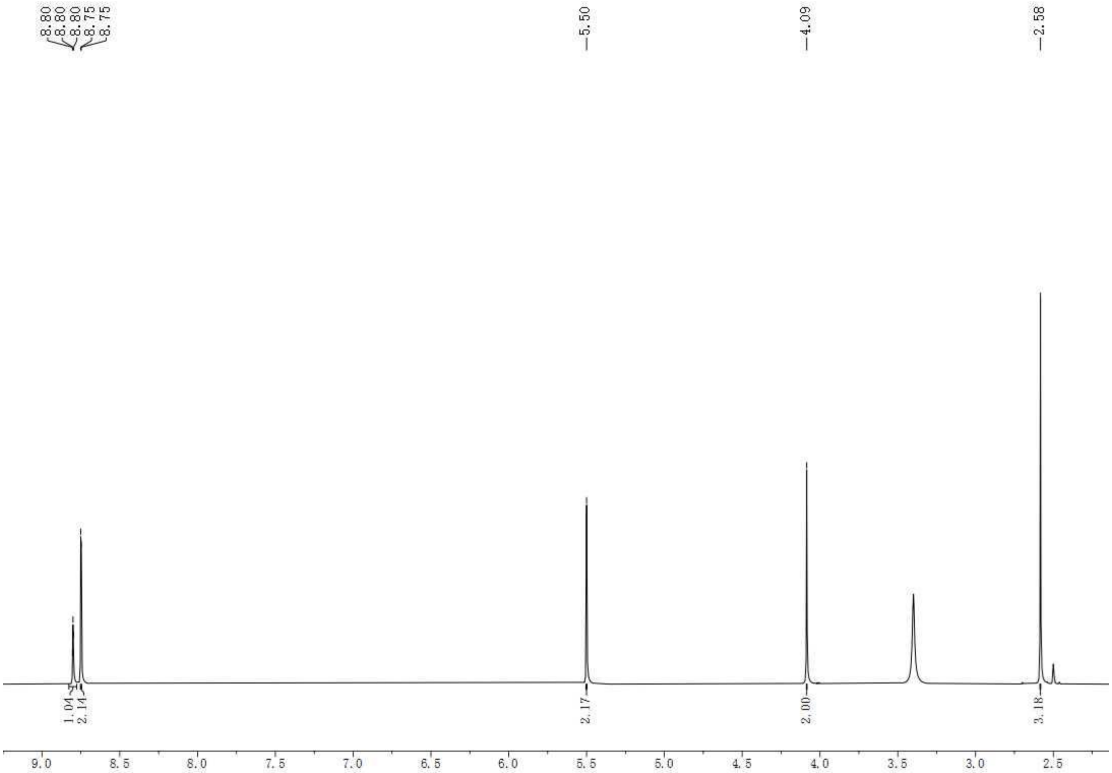
Supplementary Figure 52. ^{13}C NMR (150 MHz, MeOD) of 8.



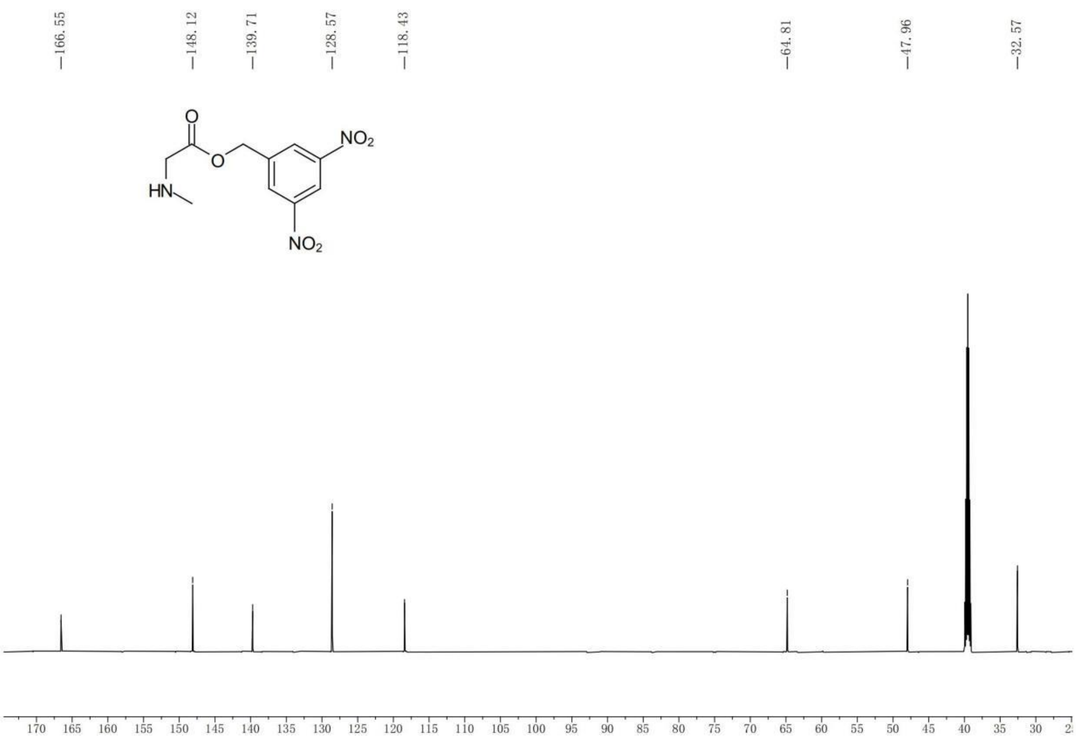
Supplementary Figure 53. The mass spectrum of 8.



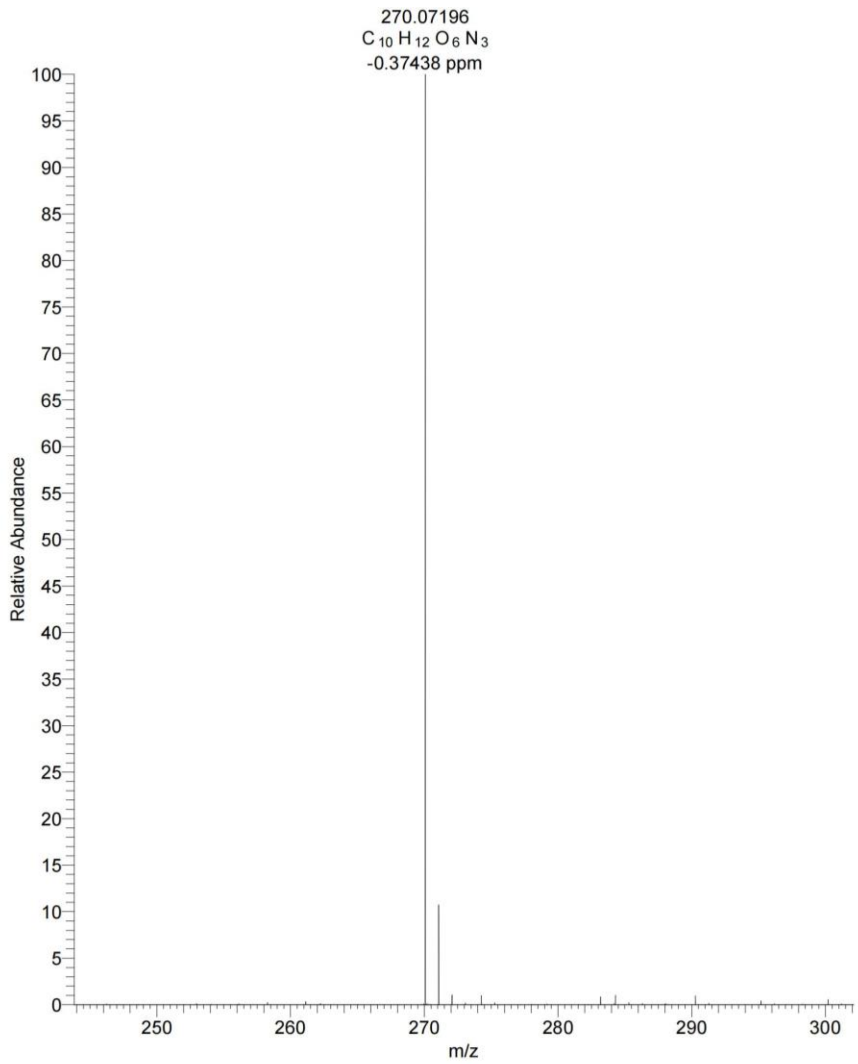
Supplementary Figure 54. ^{1}H NMR (600 MHz, DMSO-d_6) of 9.



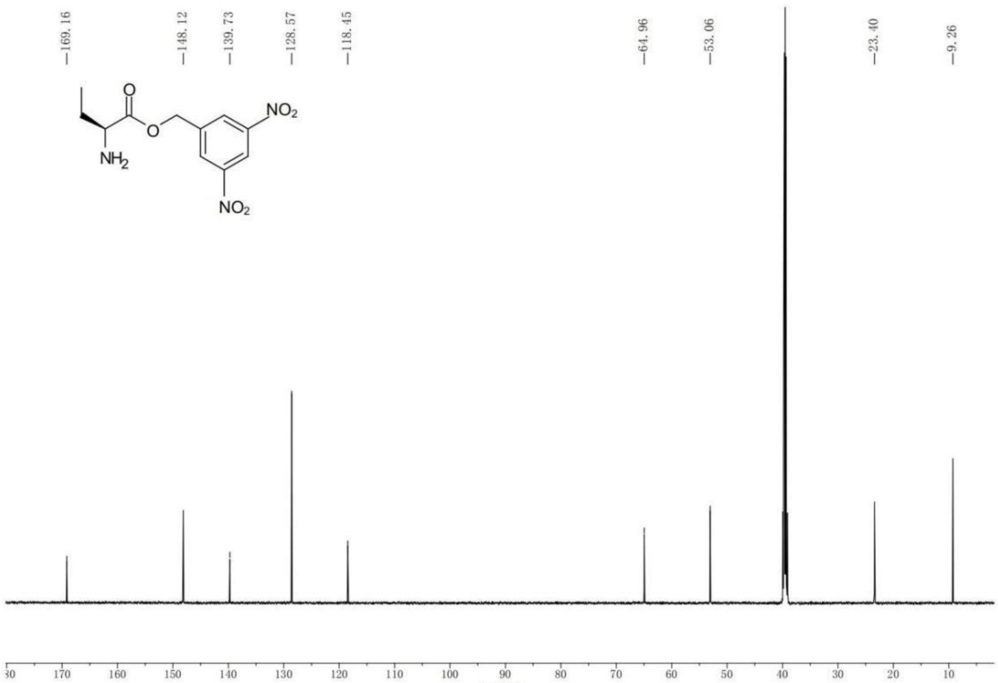
Supplementary Figure 55. ^{13}C NMR (150 MHz, DMSO-d_6) of 9.



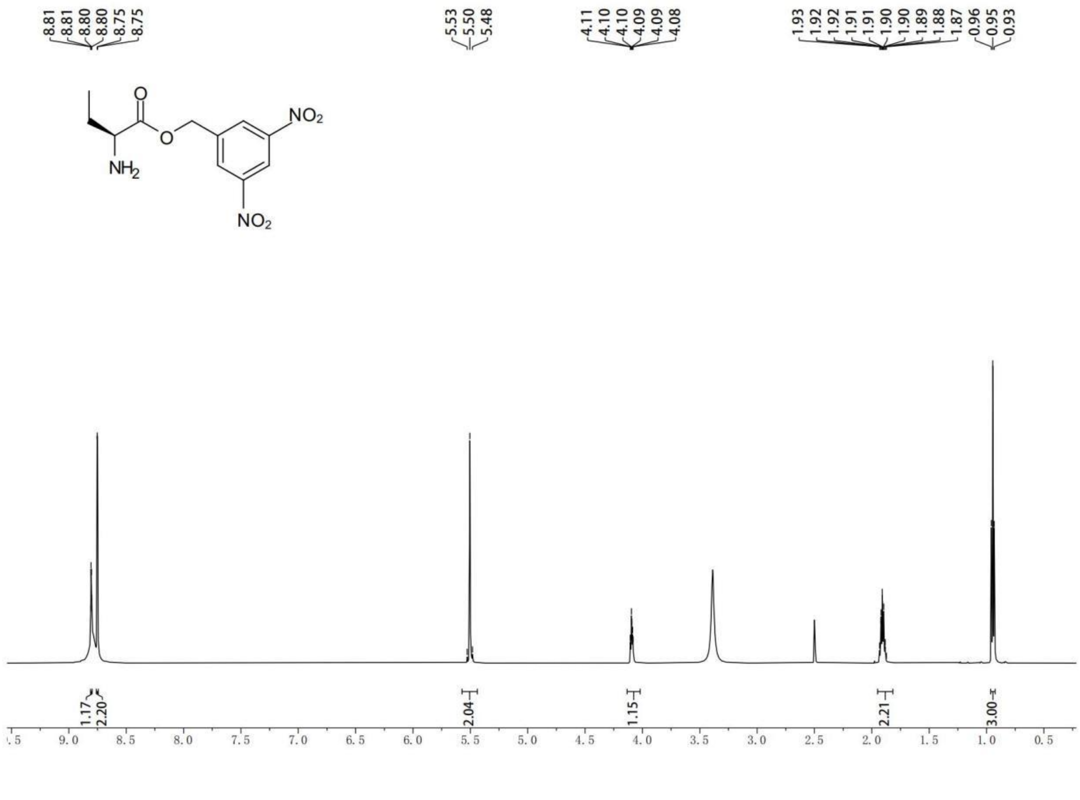
Supplementary Figure 56. The mass spectrum of 9.



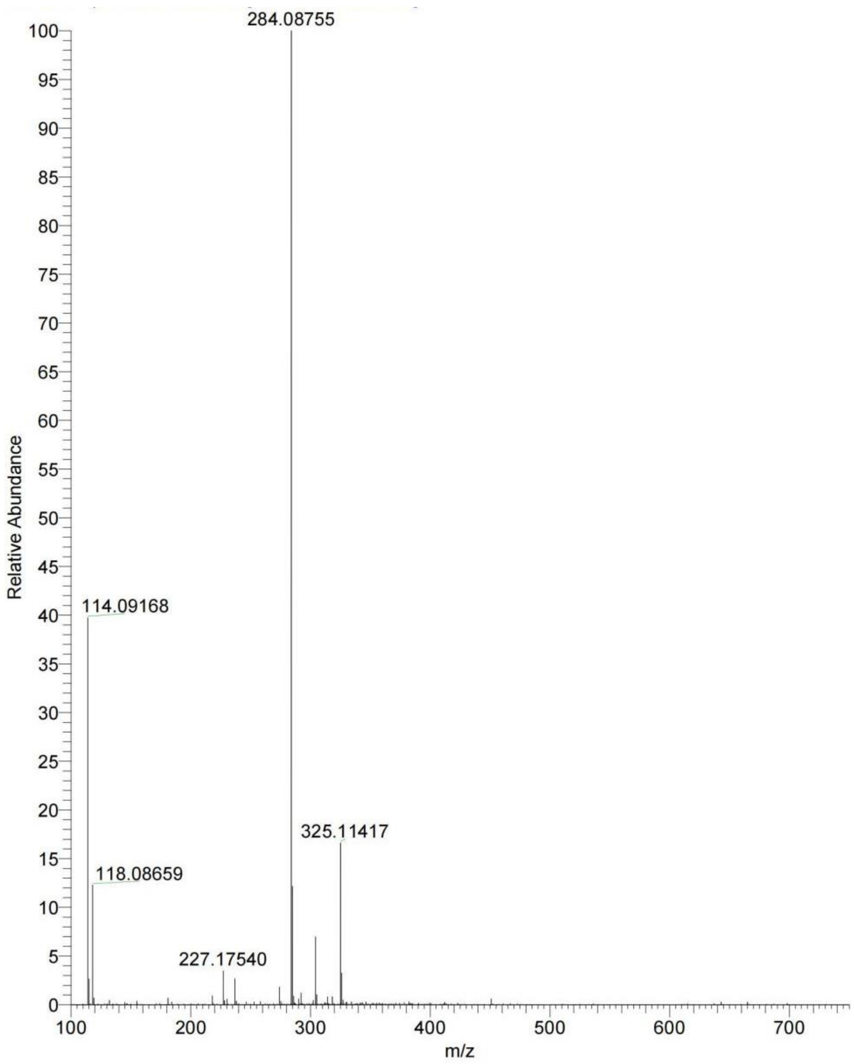
Supplementary Figure 57. ^{1}H NMR (600 MHz, DMSO-d_6) of 10.



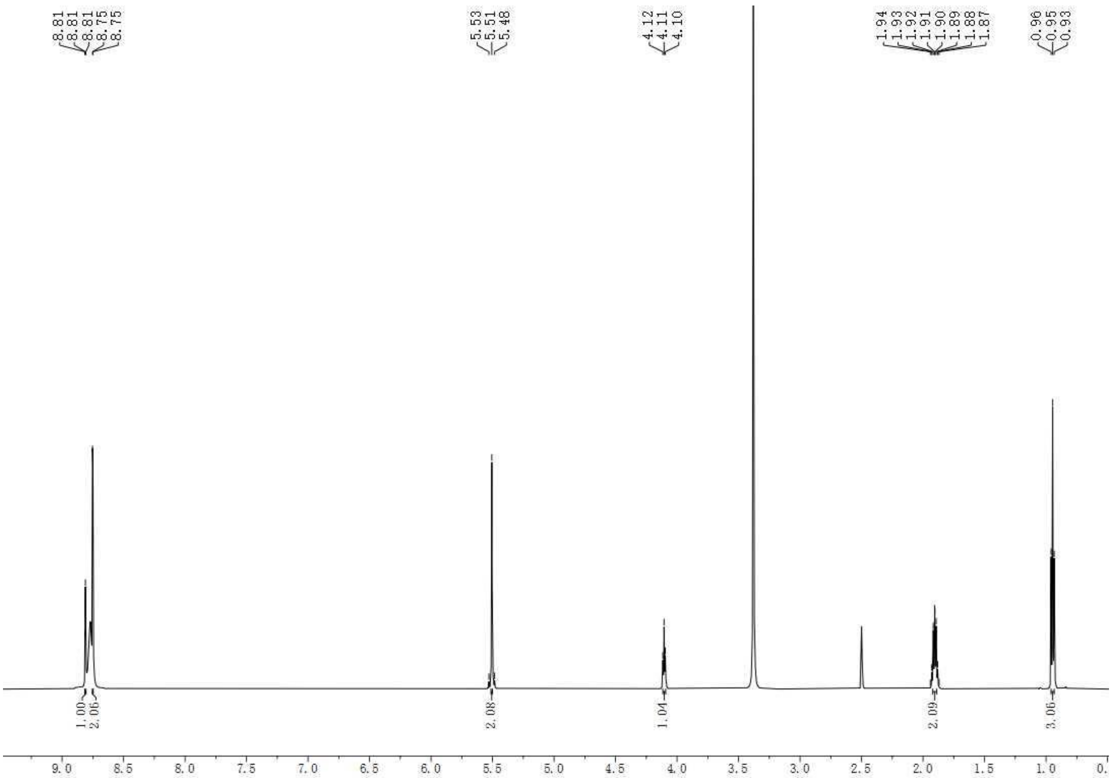
Supplementary Figure 58. ^{13}C NMR (150 MHz, DMSO-d_6) of 10.



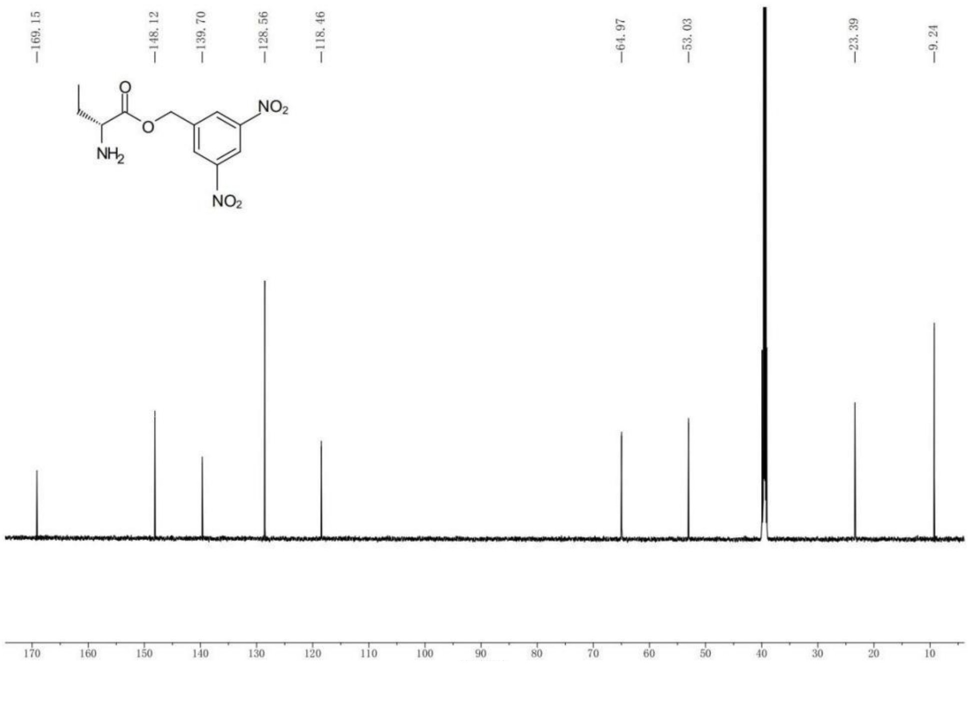
Supplementary Figure 59. The mass spectrum of 10.



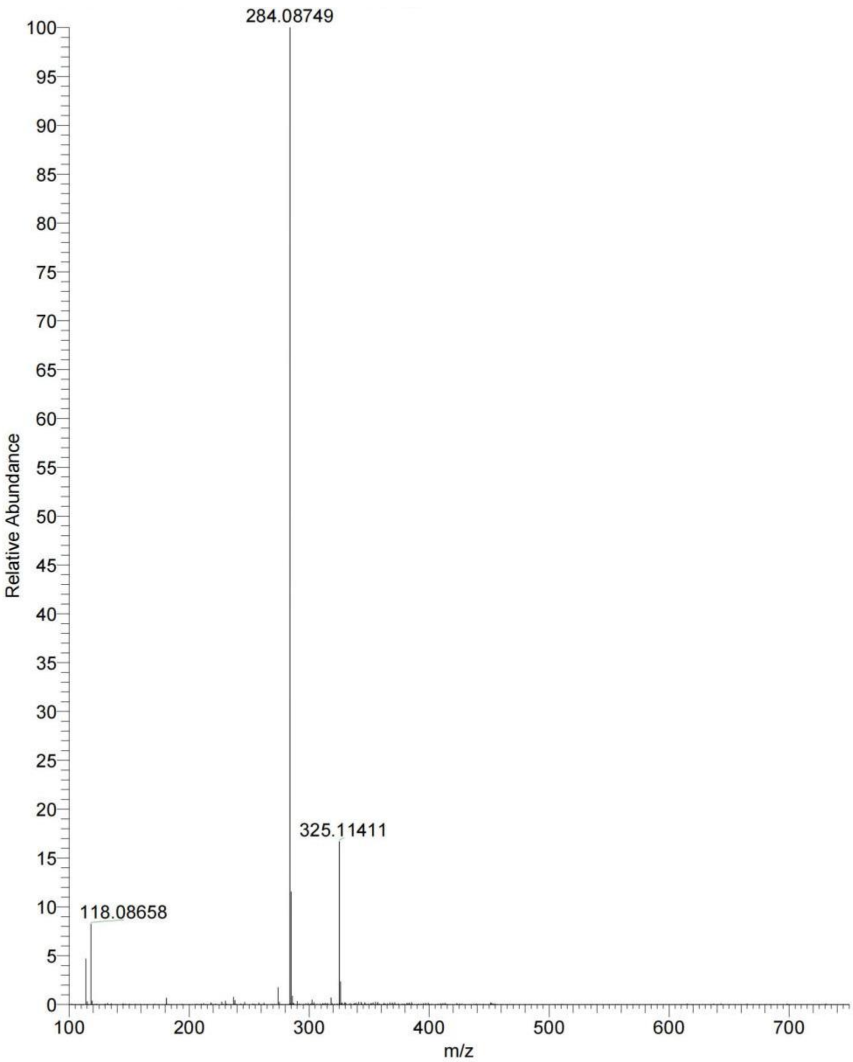
Supplementary Figure 60. ^{1}H NMR (600 MHz, DMSO-d_6) of 11.



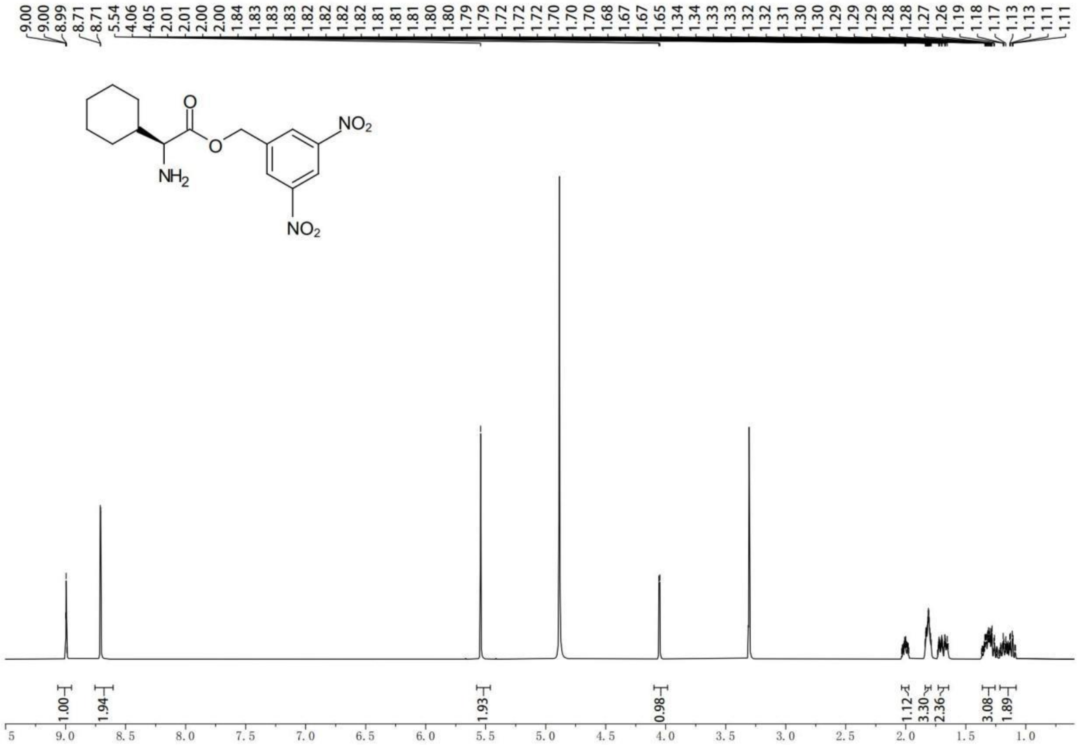
Supplementary Figure 61. ^{13}C NMR (150 MHz, DMSO-d_6) of 11.



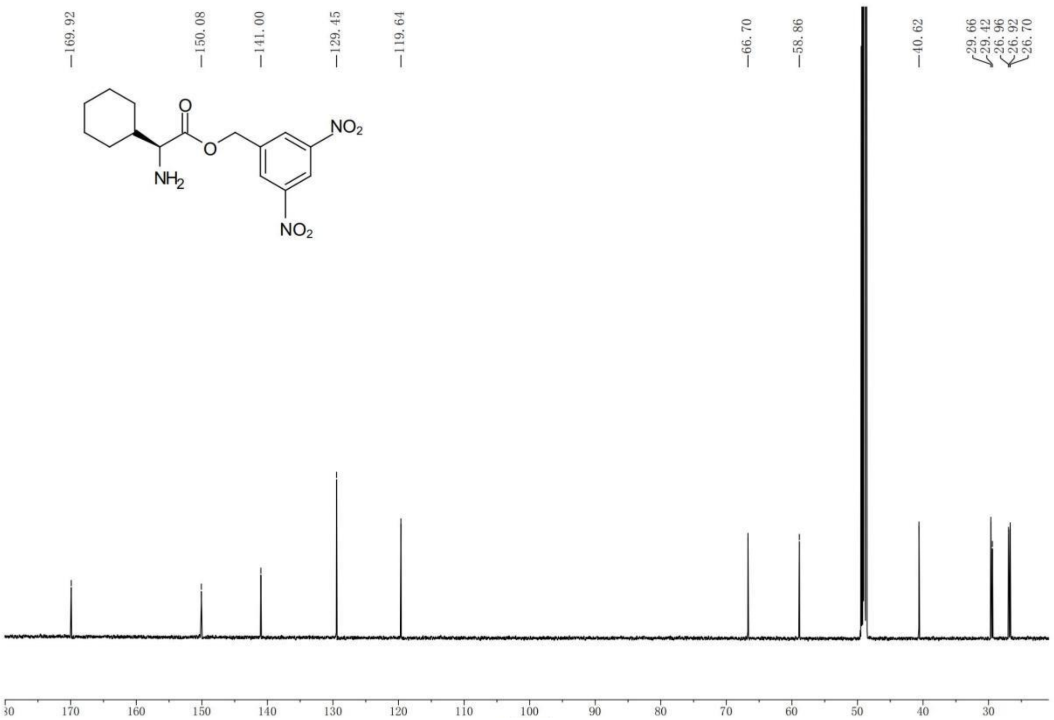
Supplementary Figure 62. The mass spectrum of 11.



Supplementary Figure 63. ^{1}H NMR (600 MHz, MeOD) of 12.

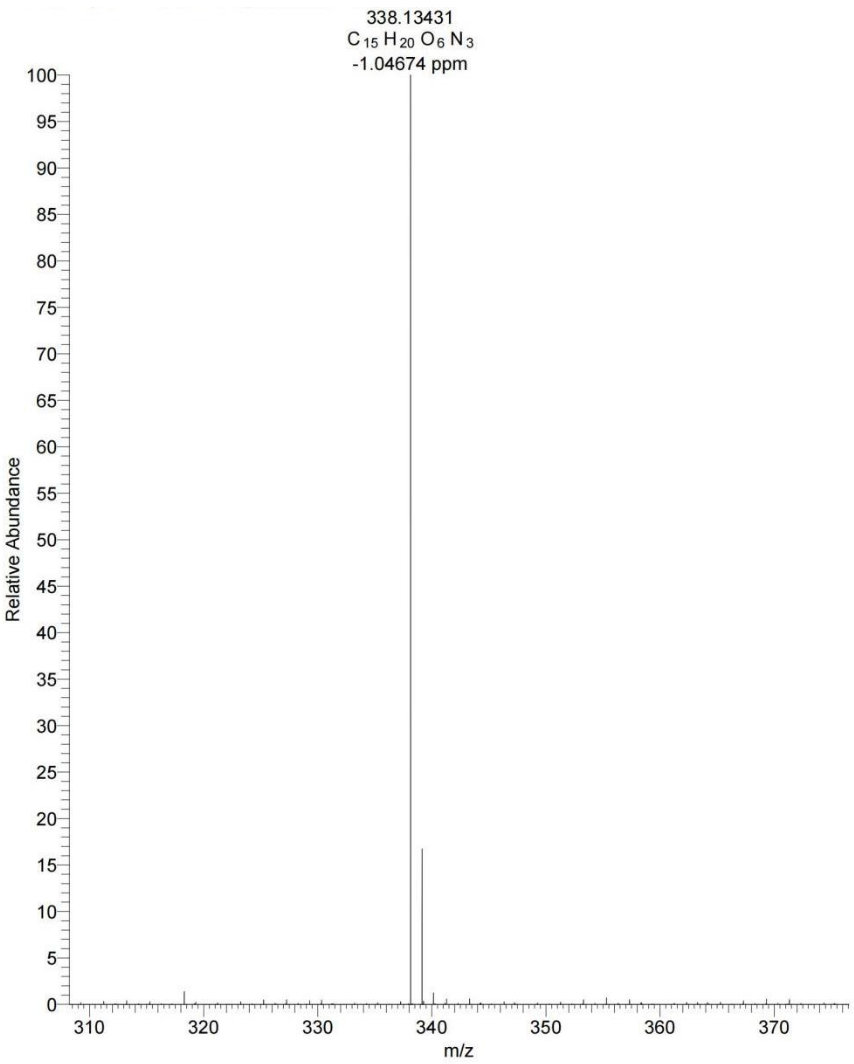


Supplementary Figure 64. ^{13}C NMR (150 MHz, MeOD) of 12.



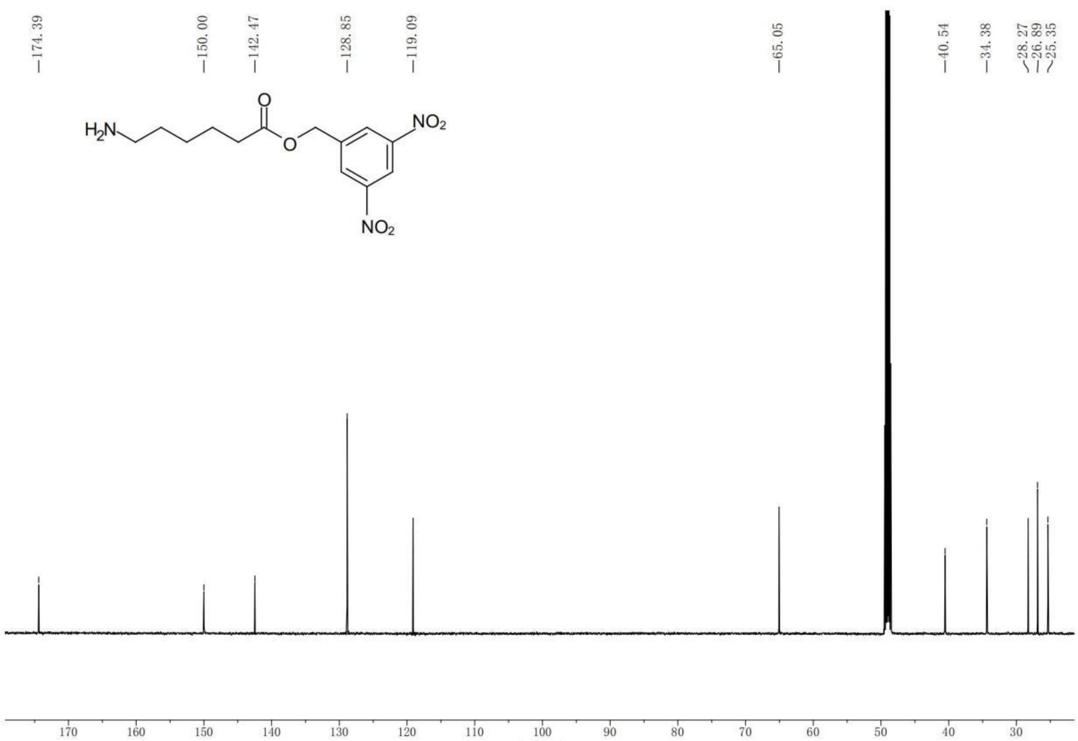
.

Supplementary Figure 65. The mass spectrum of 12.

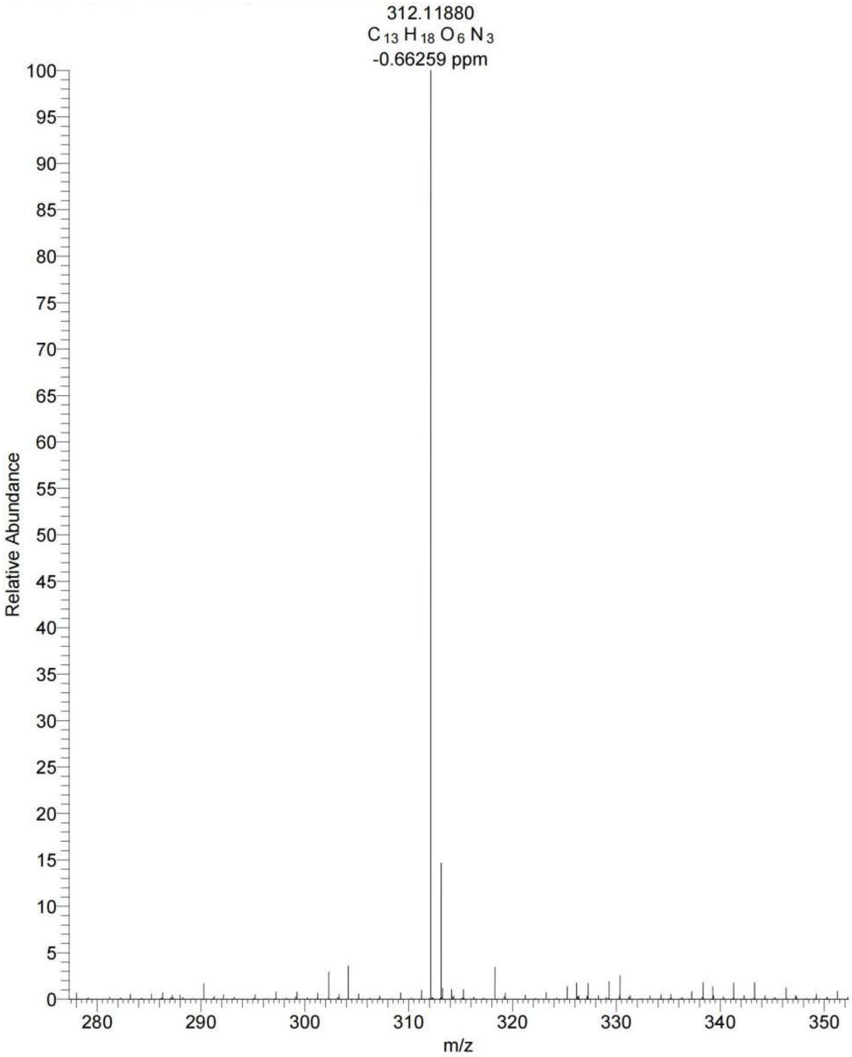


Supplementary Figure 66. ^{1}H NMR (600 MHz, MeOD) of 13.

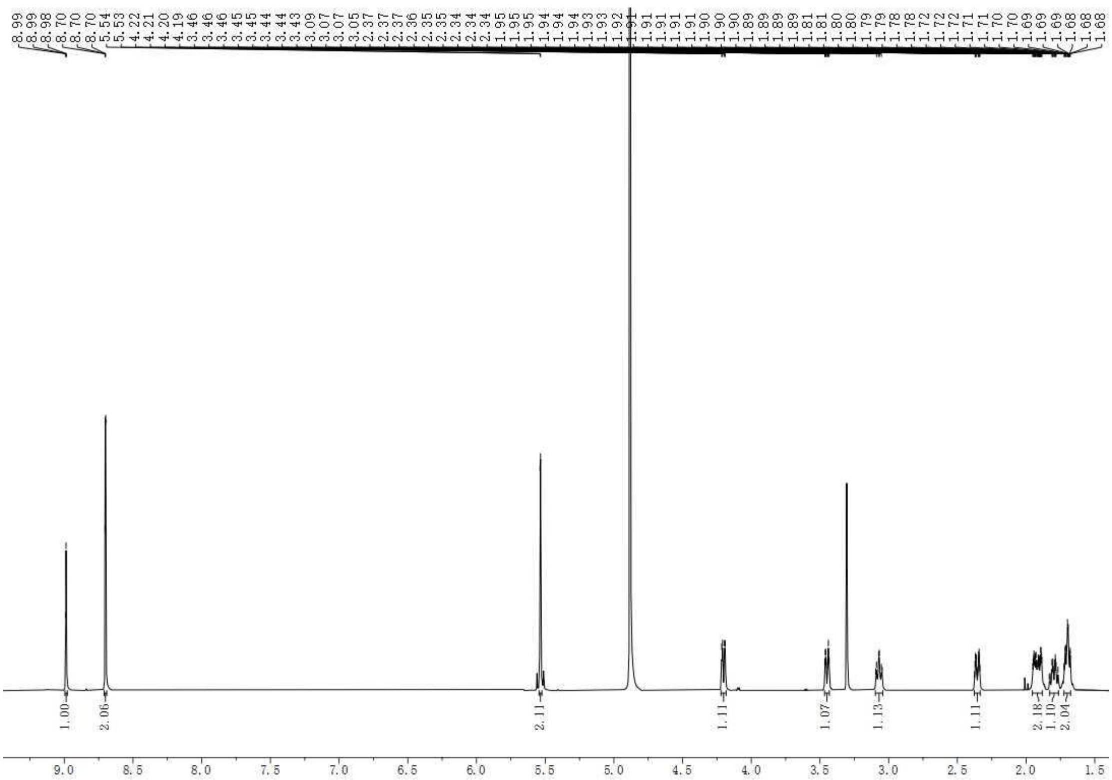
Supplementary Figure 67. ^{13}C NMR (150 MHz, MeOD) of 13.



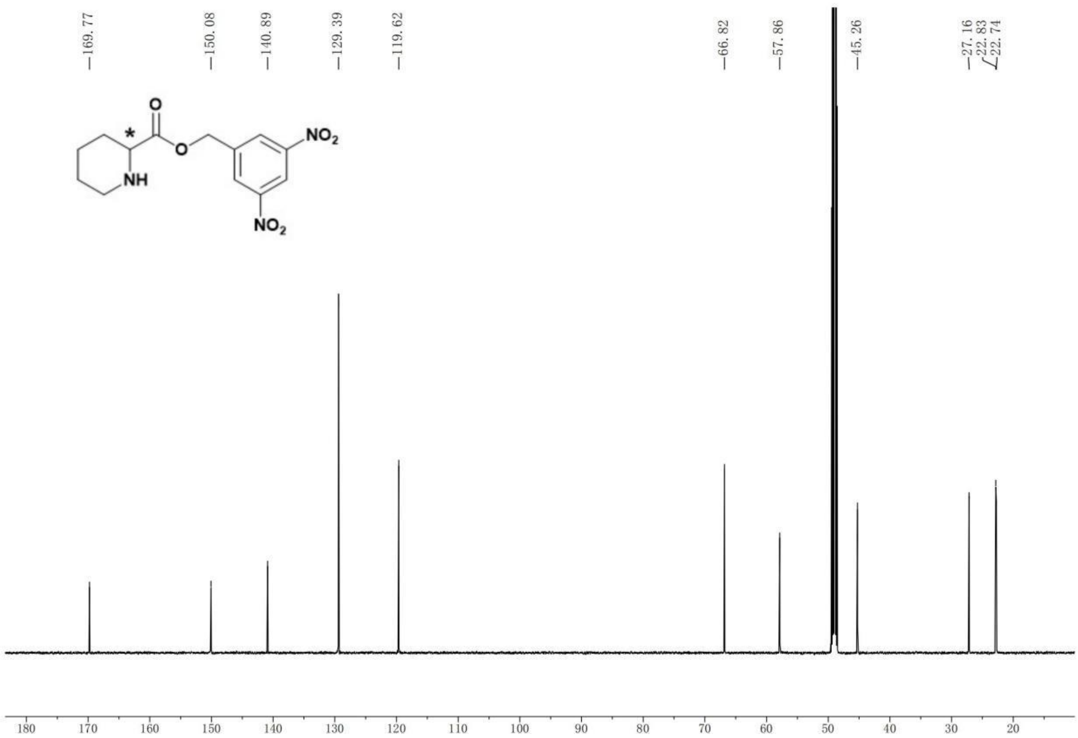
Supplementary Figure 68. The mass spectrum of 13.



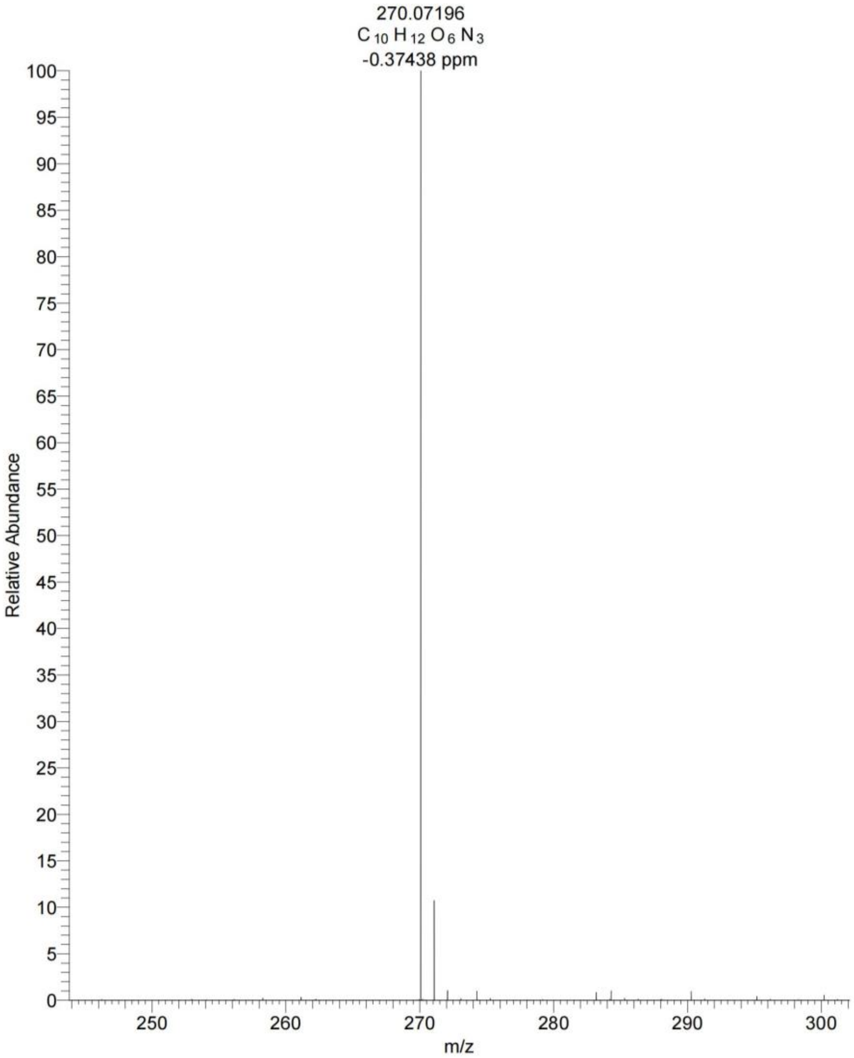
Supplementary Figure 69. ^{1}H NMR (500 MHz, MeOD) of 14.



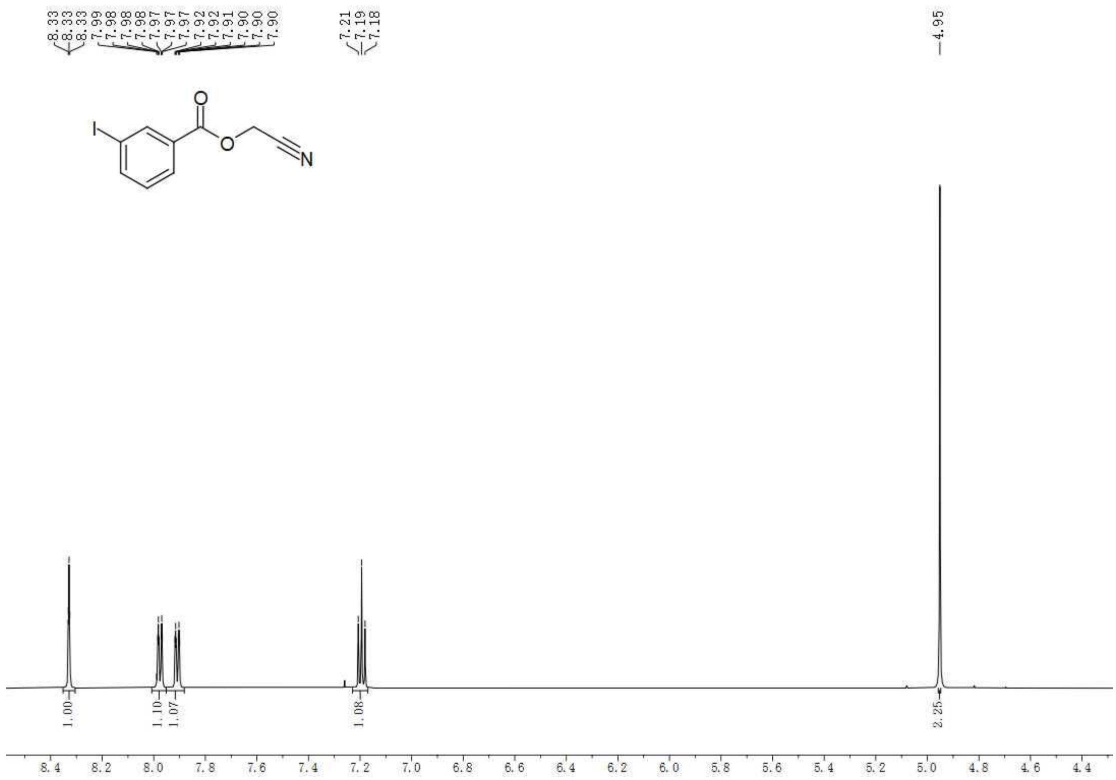
Supplementary Figure 70. ^{13}C NMR (150 MHz, MeOD) of 14.



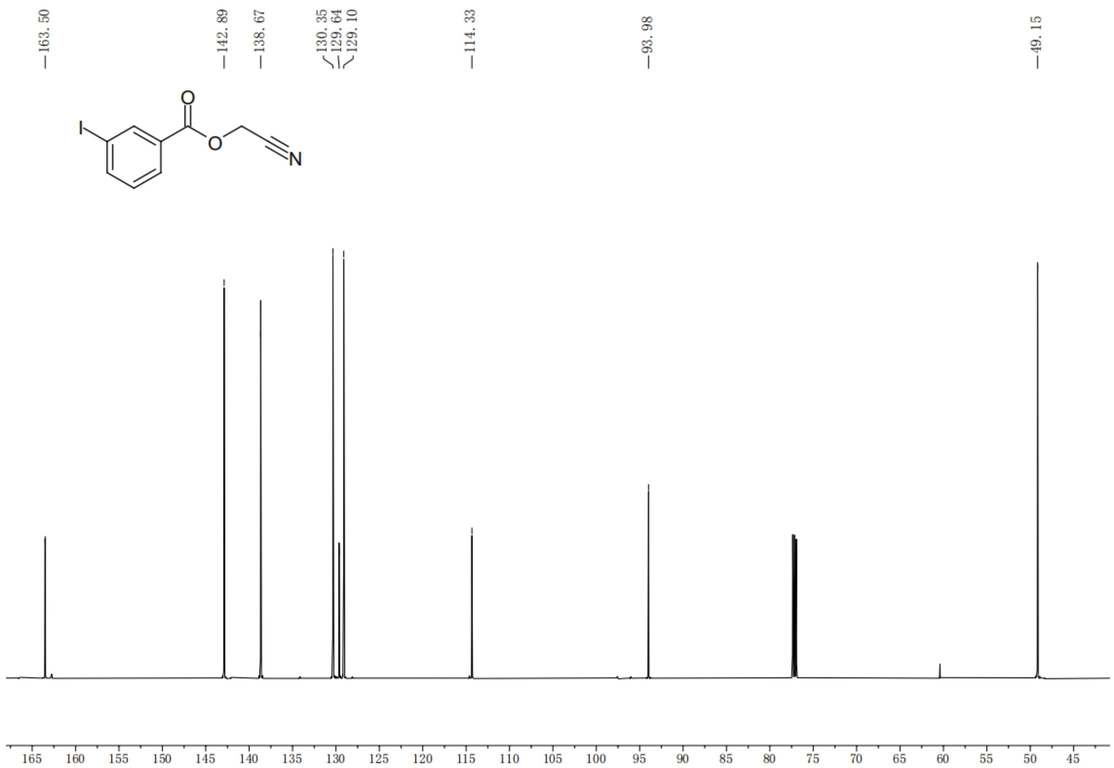
Supplementary Figure 71. The mass spectrum of 14.



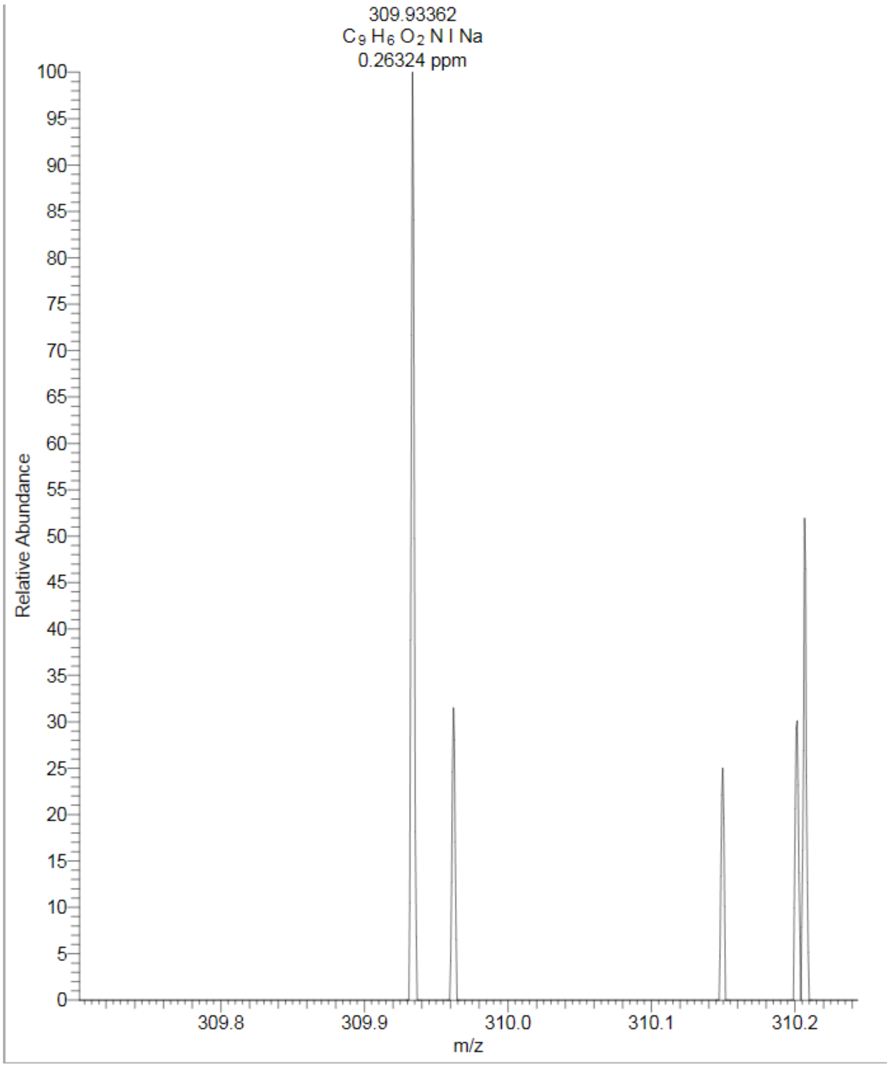
Supplementary Figure 72. ^{1}H NMR (600 MHz, $CDCl_3$) of 15.



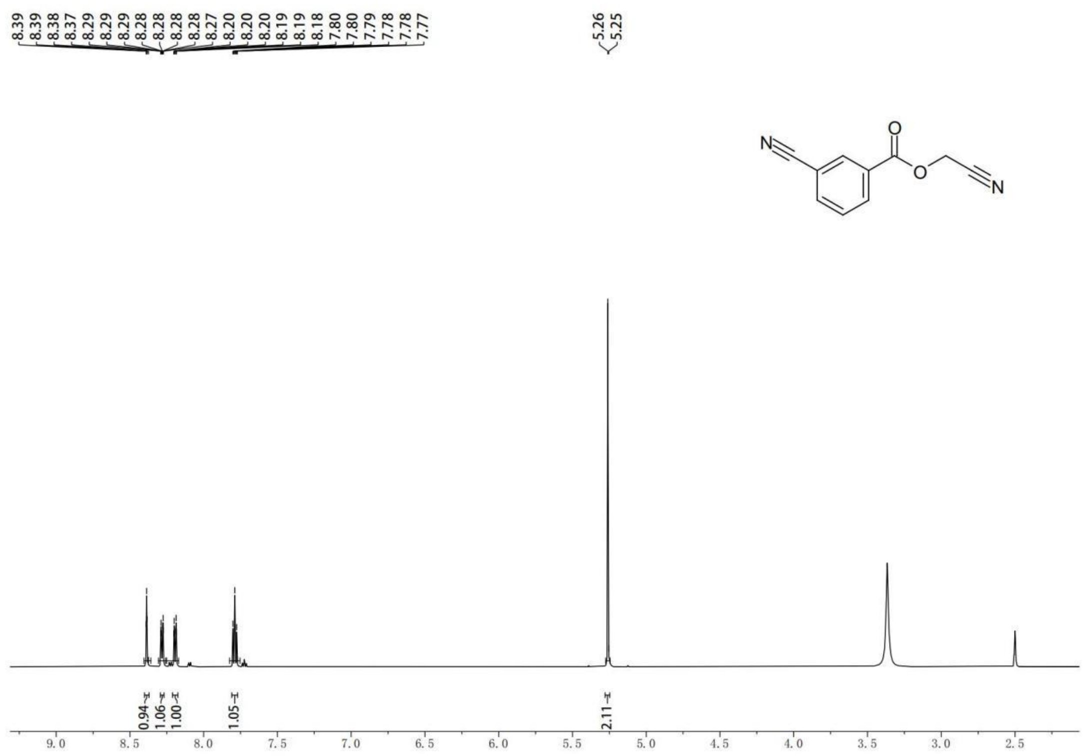
Supplementary Figure 73. ^{13}C NMR (150 MHz, $CDCl_3$) of 15.



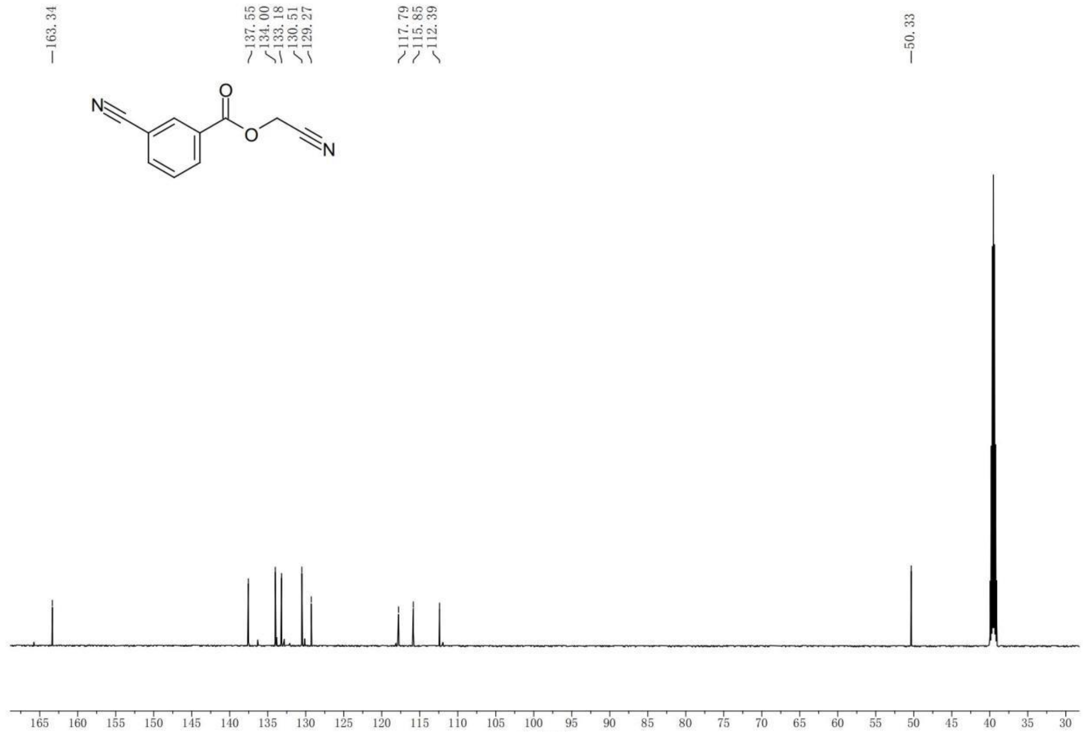
Supplementary Figure 74. The mass spectrum of 15.



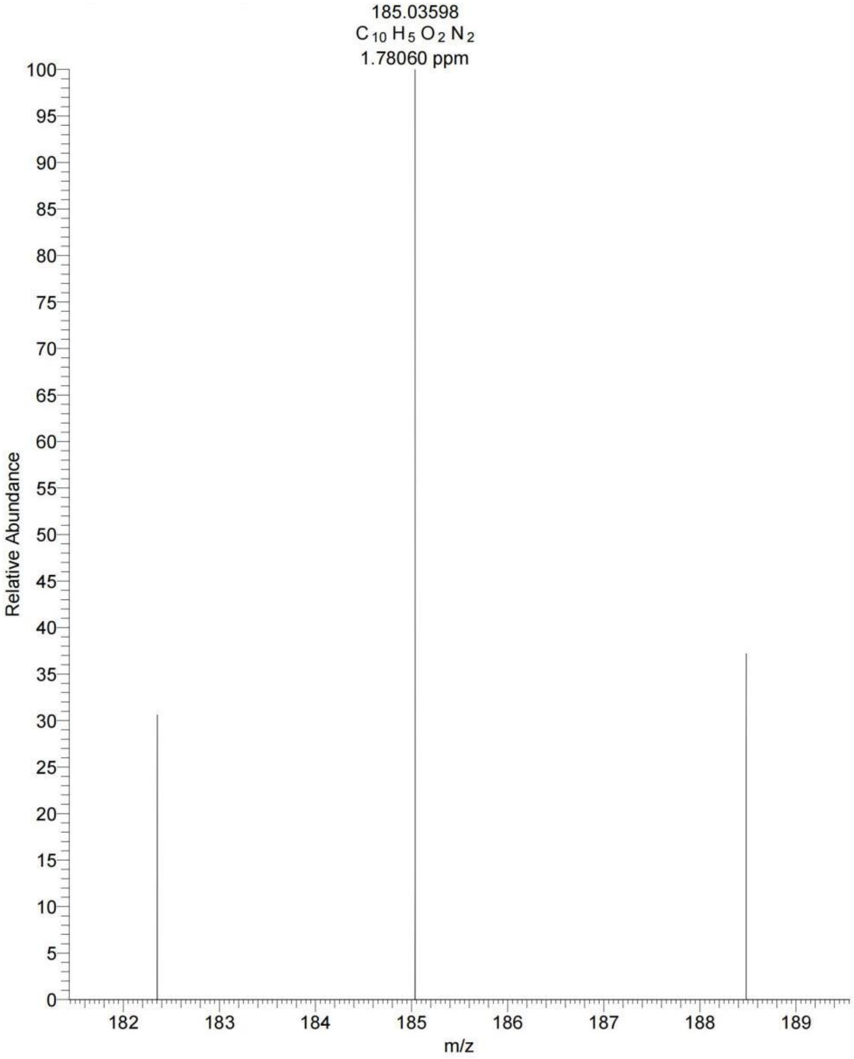
Supplementary Figure 75. ^{1}H NMR (600 MHz, DMSO-d_6) of 16.



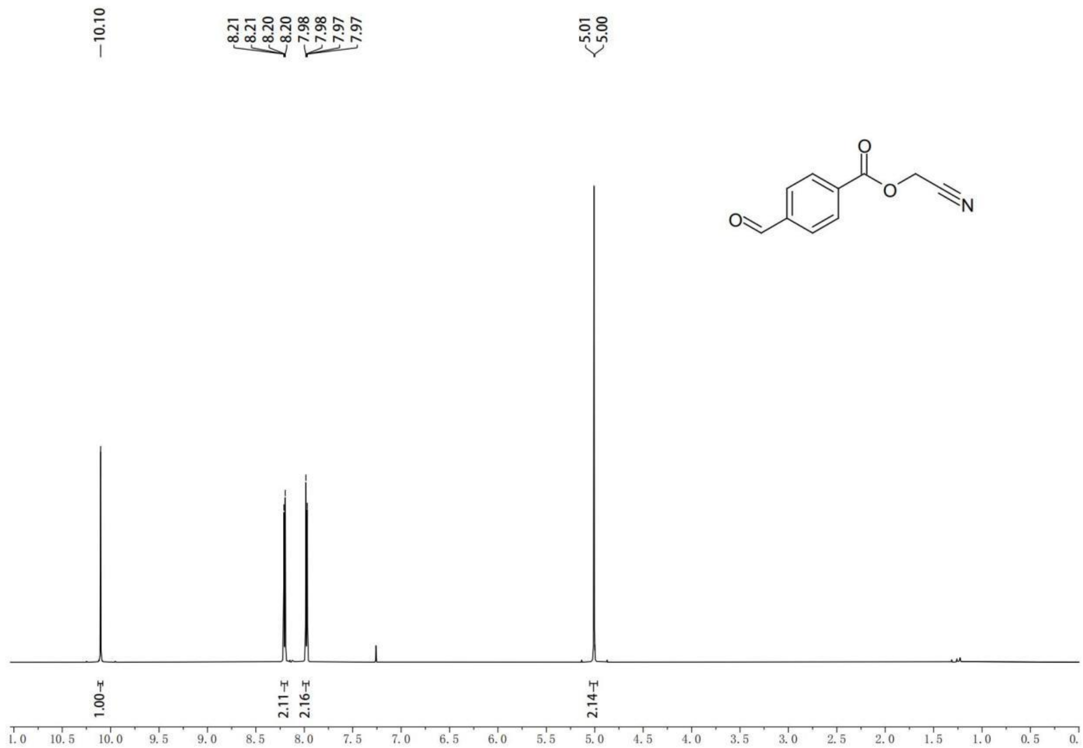
Supplementary Figure 76. ^{13}C NMR (150 MHz, DMSO-d_6) of 16.



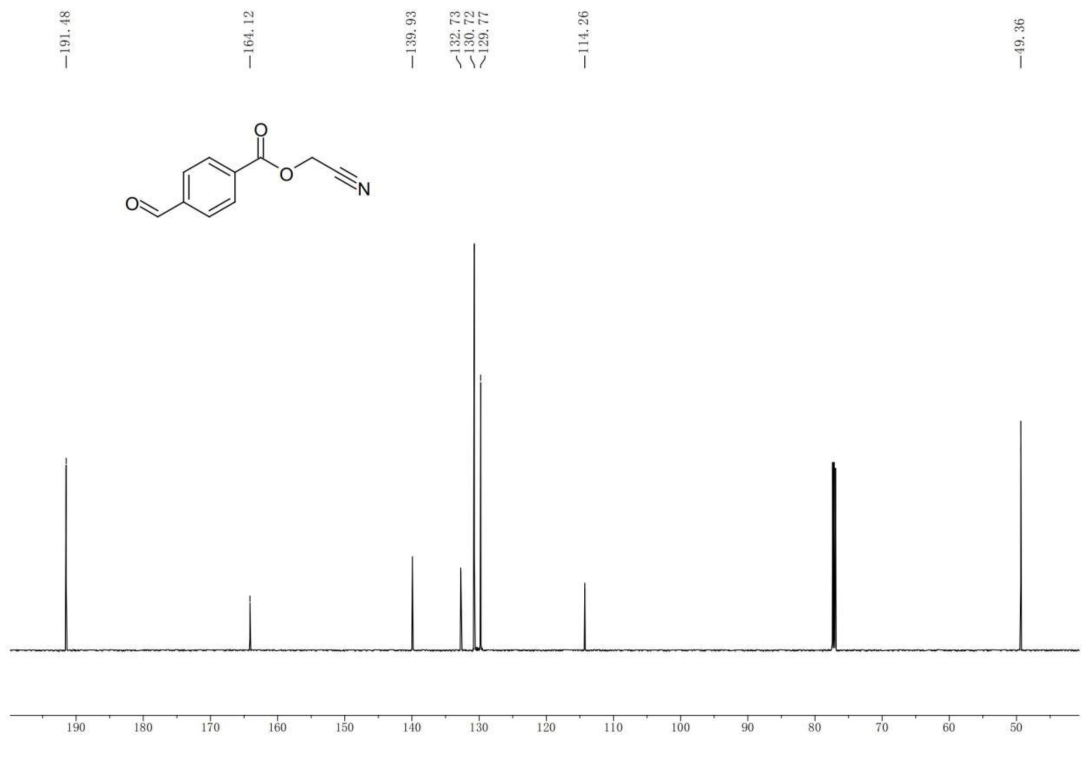
Supplementary Figure 77. The mass spectrum of 16.



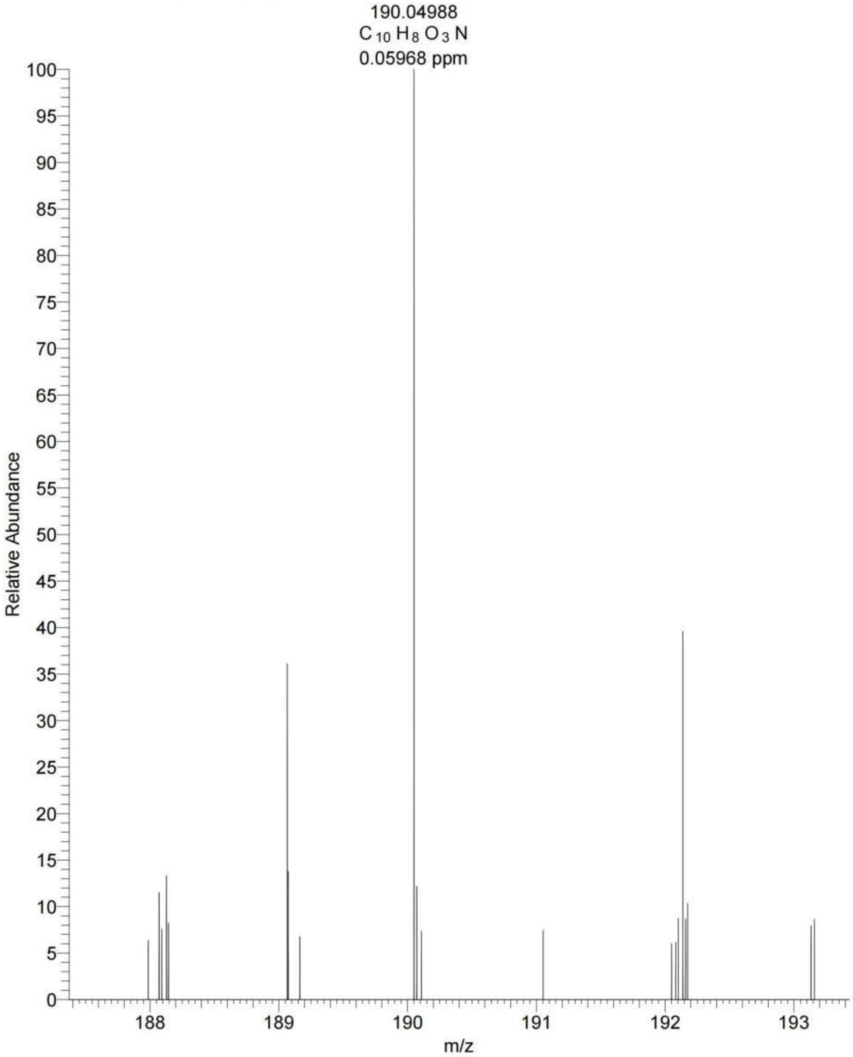
Supplementary Figure 78. ^{1}H NMR (600 MHz, $CDCl_3$) of 17.



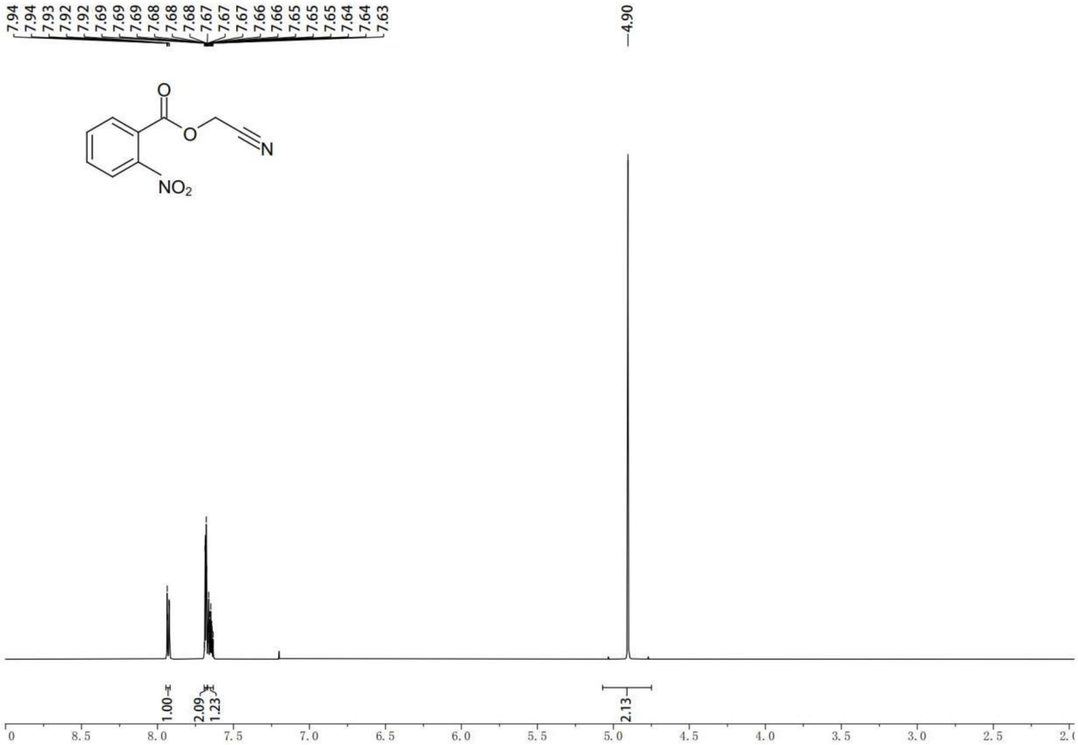
Supplementary Figure 79. ^{13}C NMR (150 MHz, $CDCl_3$) of 17.



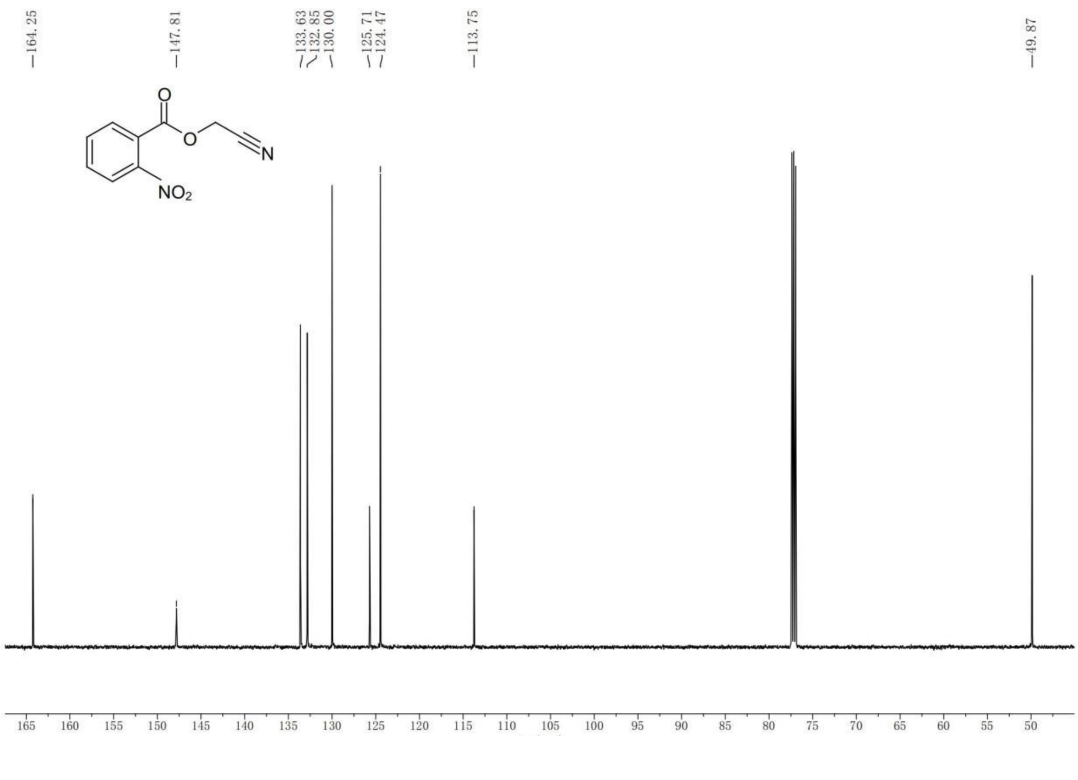
Supplementary Figure 80. The mass spectrum of 17.



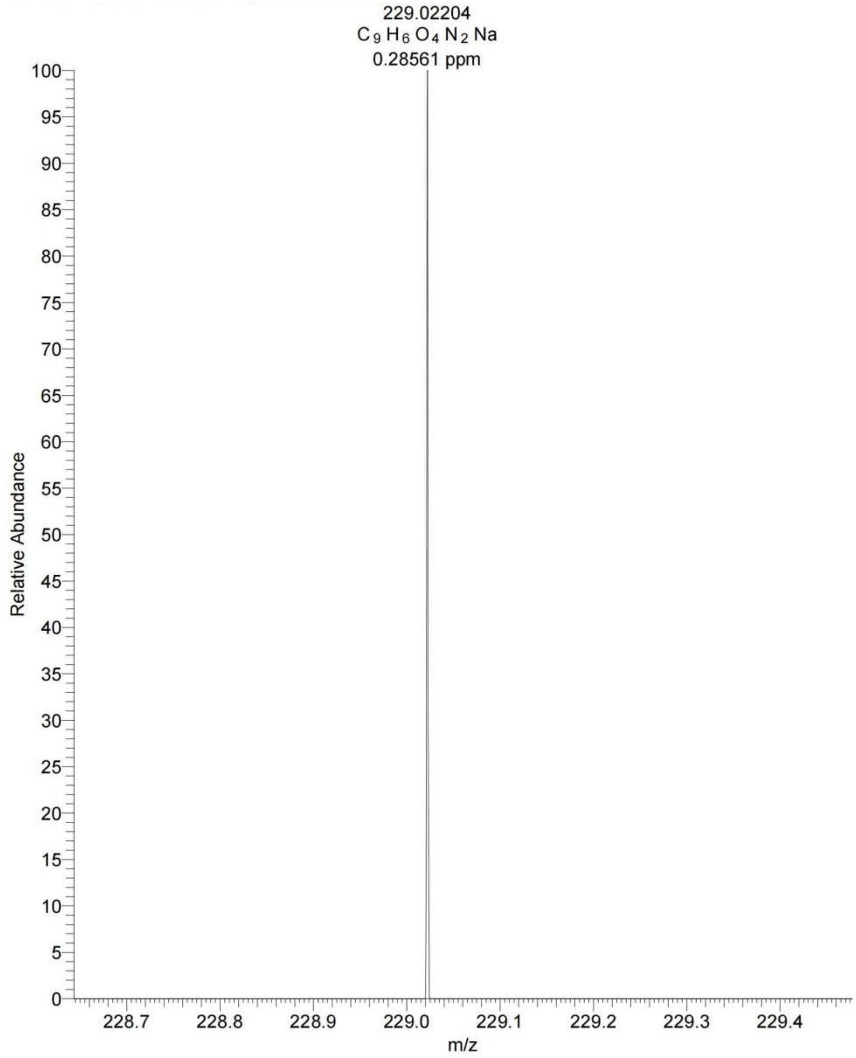
Supplementary Figure 81. ^{1}H NMR (600 MHz, $CDCl_3$) of 18.



Supplementary Figure 82. ^{13}C NMR (150 MHz, $CDCl_3$) of 18.



Supplementary Figure 83. The mass spectrum of 18.



Reference

1. H. Saito, D. Kourouklis and H. Suga, *Embo j*, 2001, **20**, 1797-1806.

2. D. Kourouklis, H. Murakami and H. Suga, *Methods*, 2005, **36**, 239-244.

3. Y. Goto, T. Katoh and H. Suga, *Nat Protoc.*, 2011, **6**, 779-790.

4. J. Lee, K. E. Schwieter, A. M. Watkins, D. S. Kim, H. Yu, K. J. Schwarz, J. Lim, J.Coronado, M. Byrom, E. V. Anslyn, A. D. Ellington, J. S. Moore and M. C. Jewett, *Nat Commun.*, 2019, **10**, 5097.