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Identification of inhibitors of UDP-galactopyranose mutase via combinatorial *in situ* screening

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Electronic Supplementary Information

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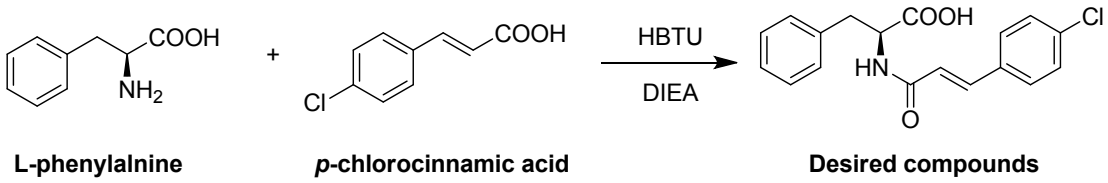
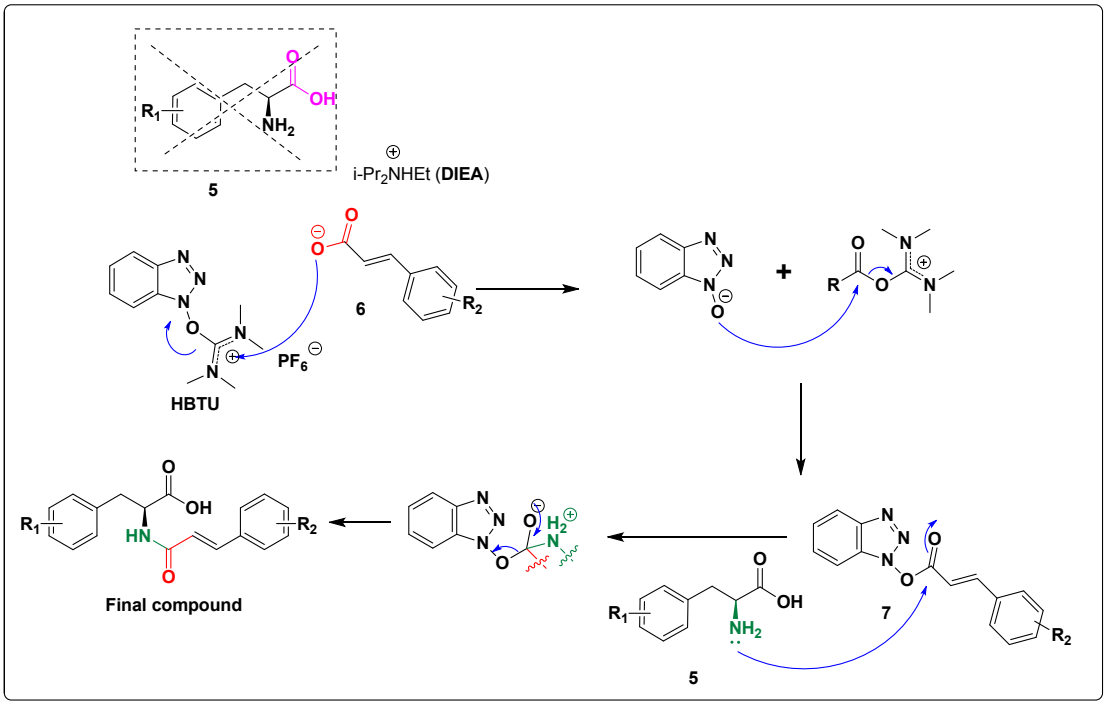
Combinatorial *in situ* FP screening



Scheme S1 A model study of L-phenylalnine and *p*-chlorocinnamic acid



Scheme S2 Acid activation and amide bond formation with HBTU

TLC monitor

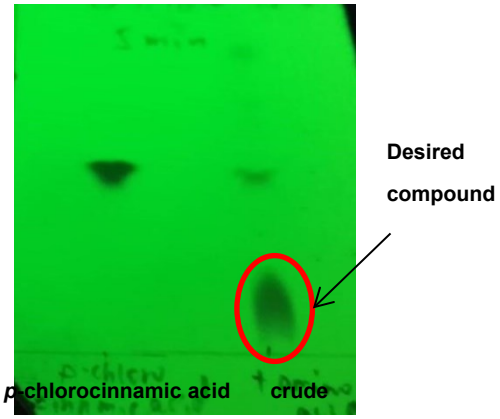
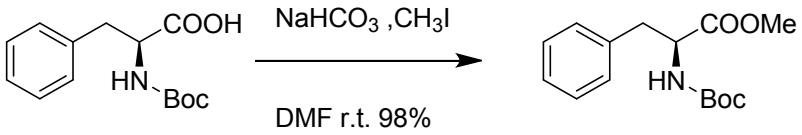


Fig. S1 Trace of TLC 2h (left side), 5min (right side)

Kinetic study by HPLC

HPLC was employed to quantify the kinetic of amide bond formation. Boc-Phe-Ome was synthesized as internal standard.

The HPLC condition as below:
Column: C18 Atlantis T3 column, 5 μm 4.6 x 250 mm;
Mobile phase: A acetonitrile 0.01% TFA/B water 0.01% TFA (45 : 55, V/V)
Wavelength: 254 nm;
Rate: 1 mL/min;
Temperature: 25°C.



Scheme S3 Synthesis of Boc-Phe-Ome

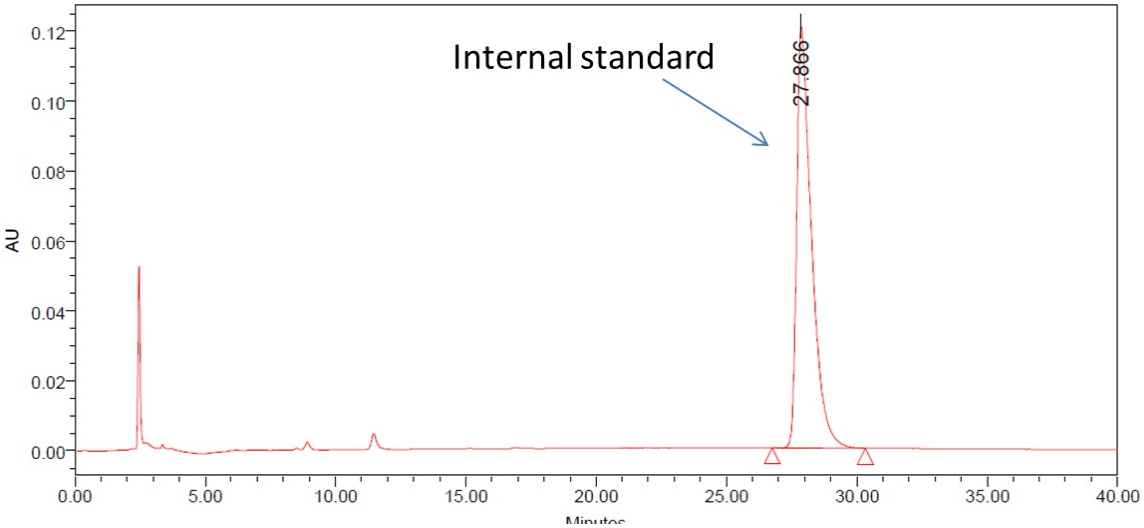
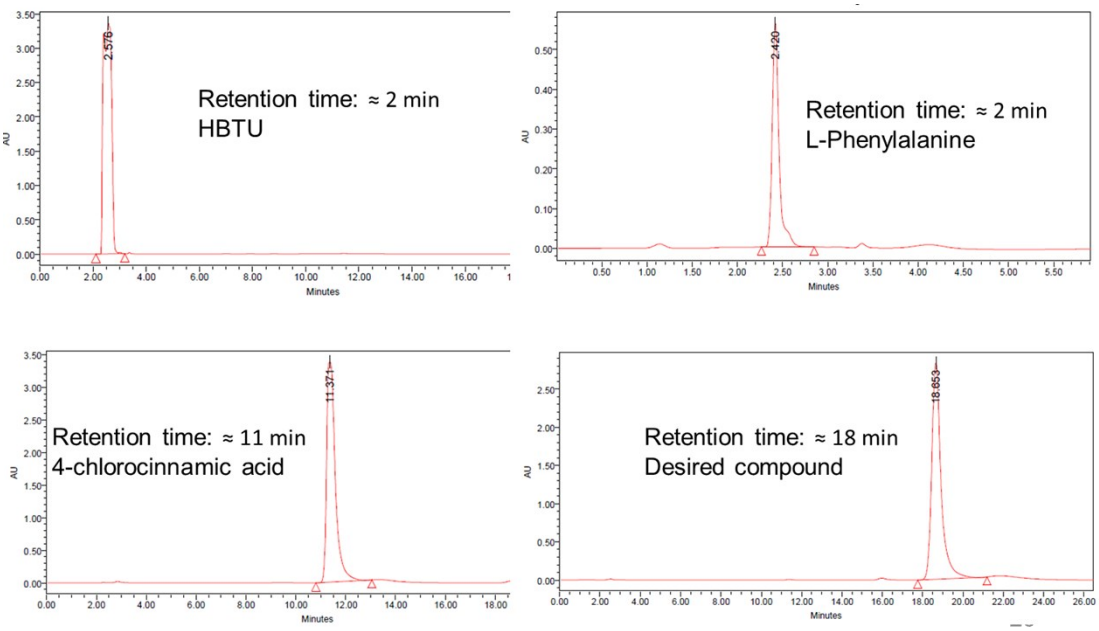


Fig. S2 HPLC of Boc-Phe-Ome



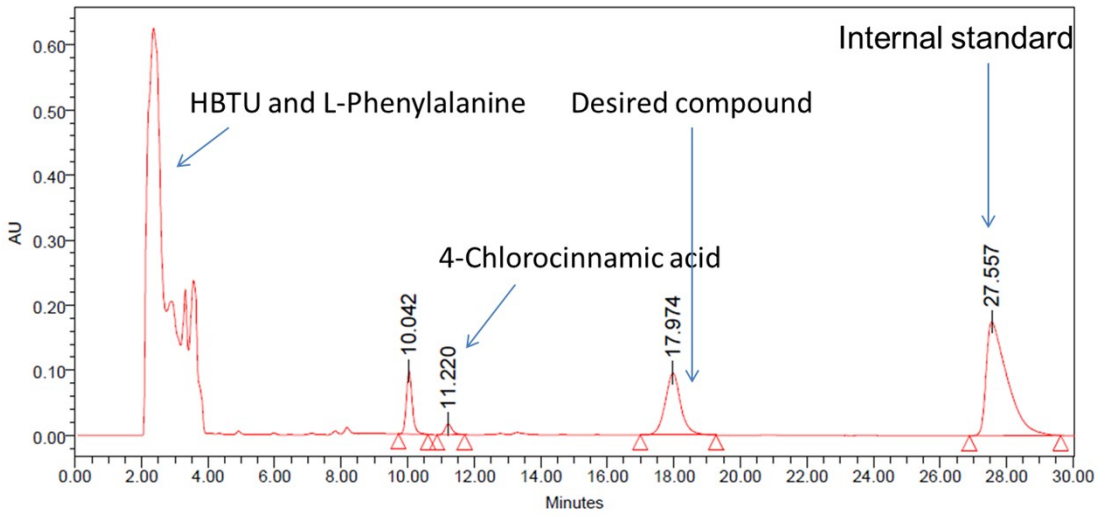
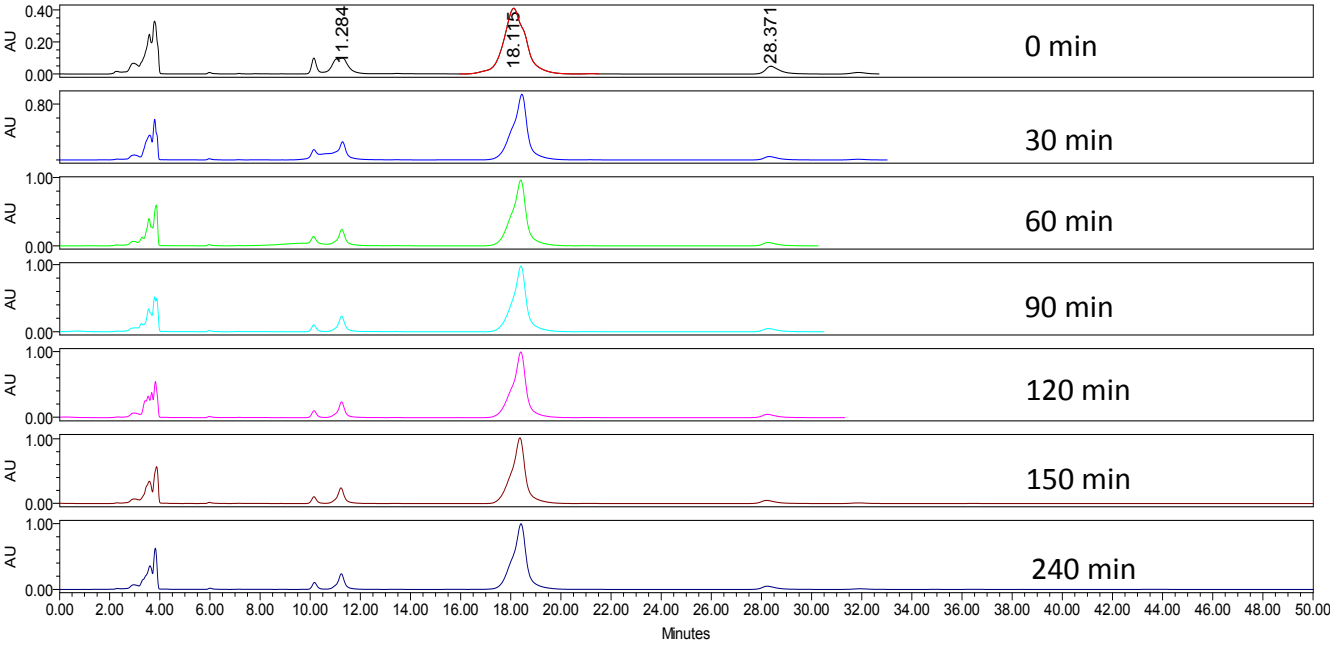


Fig. S3 HPLC chromatography



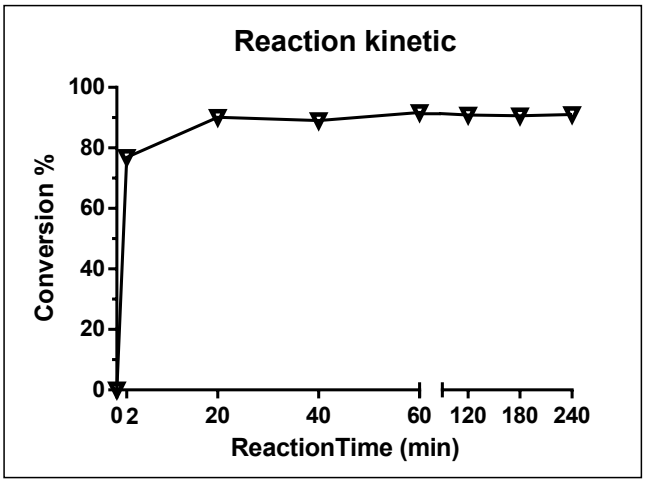


Fig. S4. Time-course of the amide bond formation.

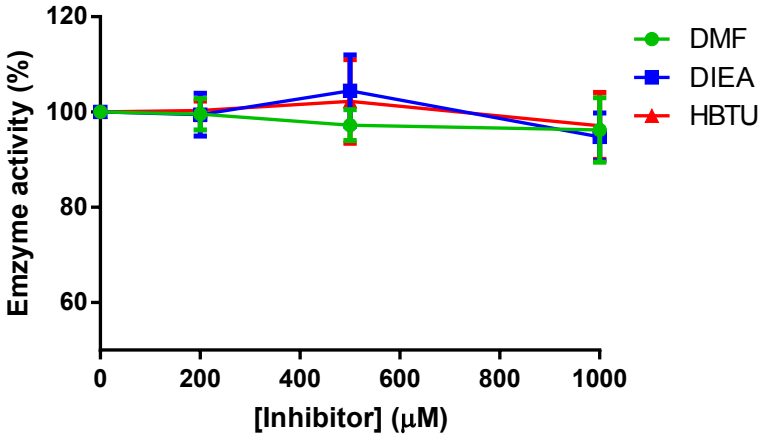


Fig. S5. Inhibitory activity of reagents involved in the in *situ* screening assay.

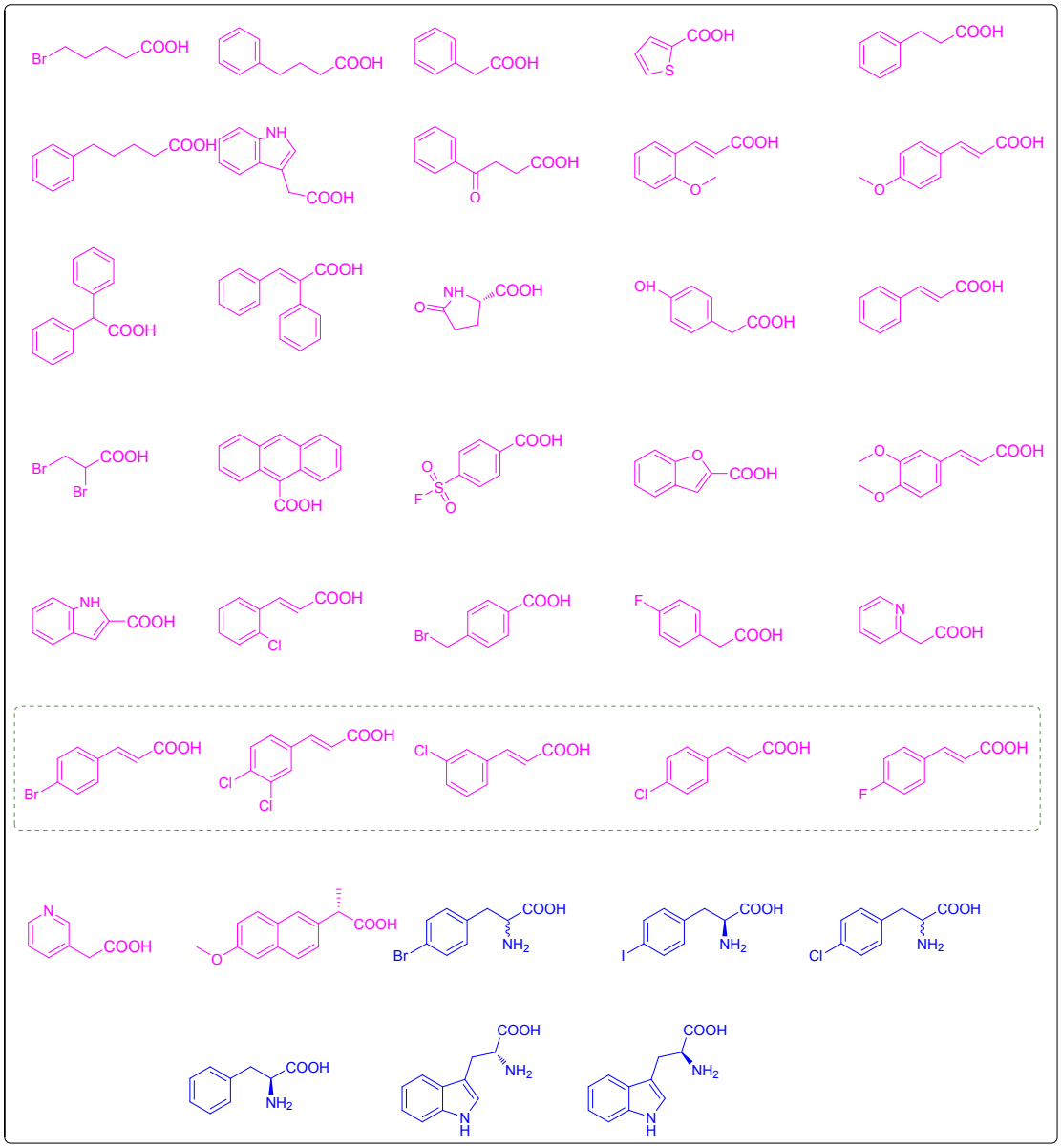
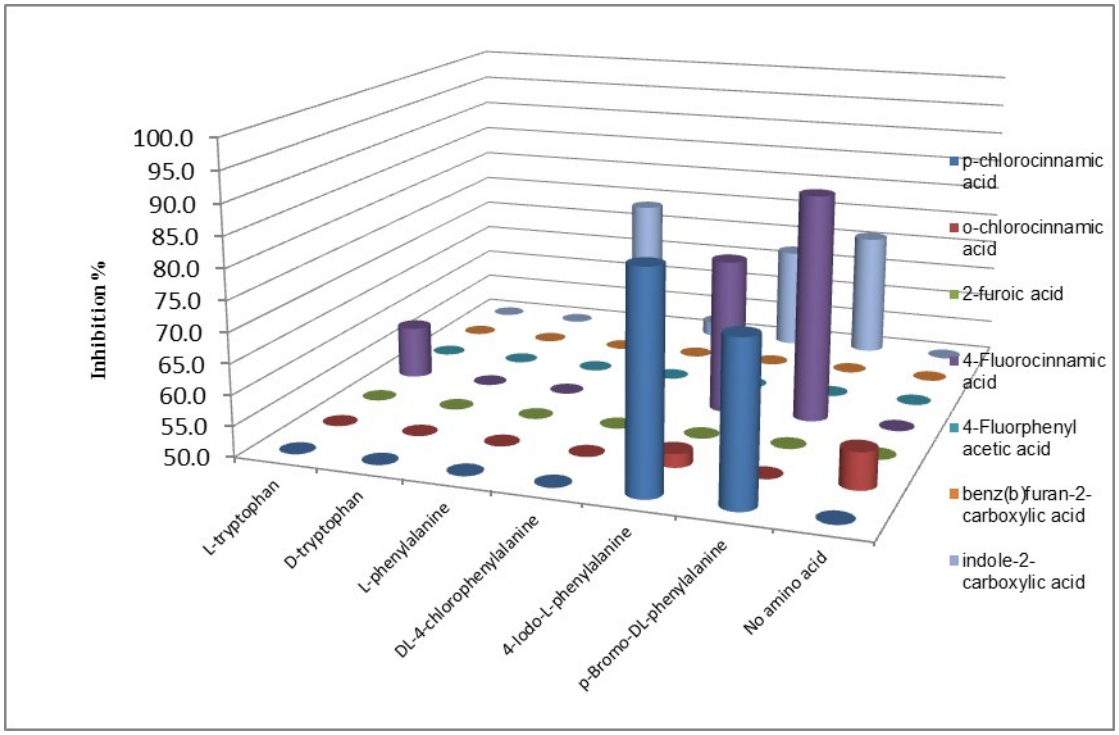


Fig. S6 Carboxylic acids (pink) and amino acids (blue) building blocks for the *in situ* screening. The best carboxylic acids were highlighted in green dash frame.



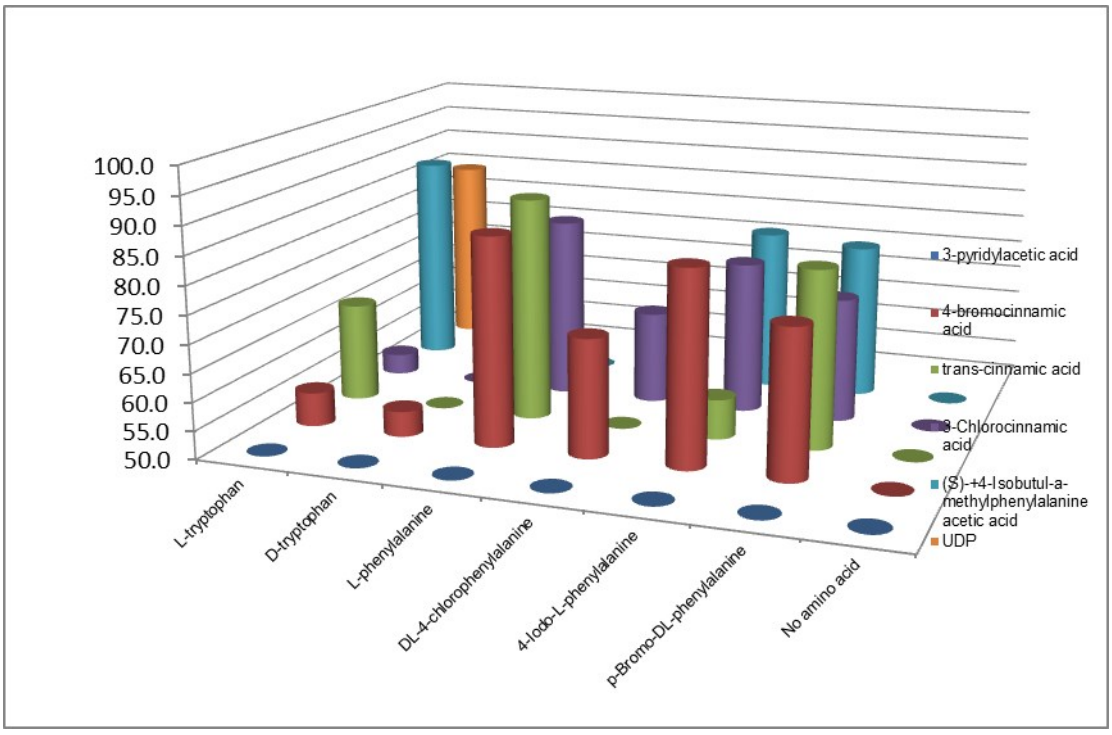
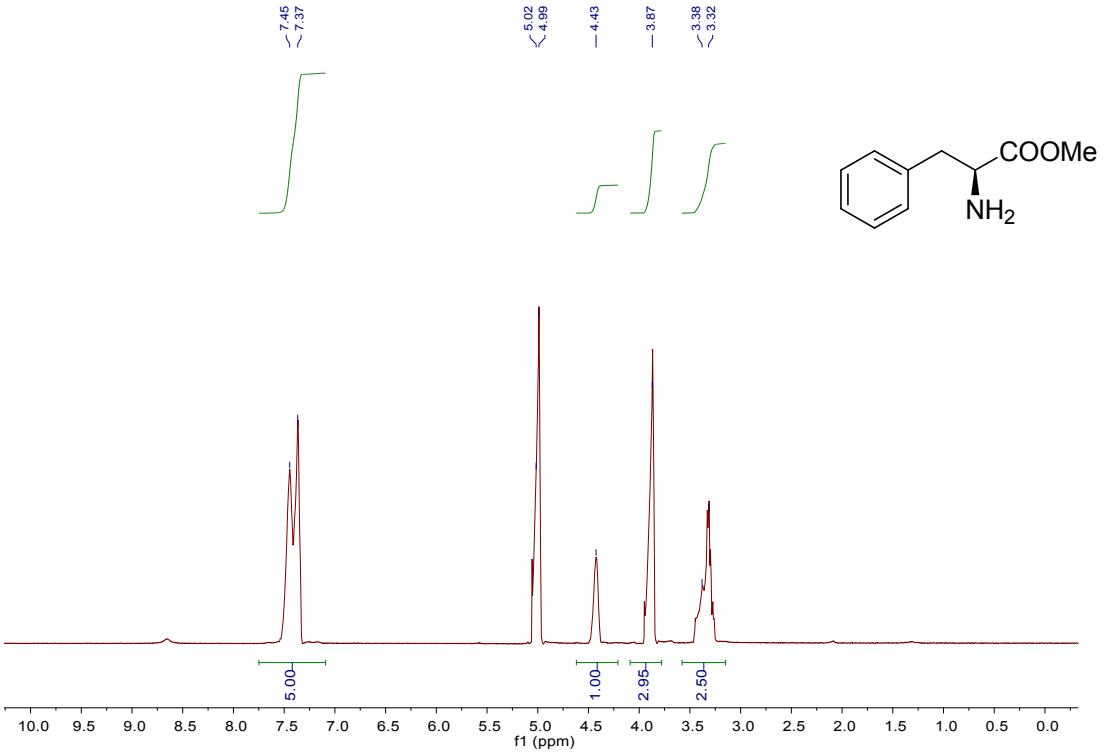
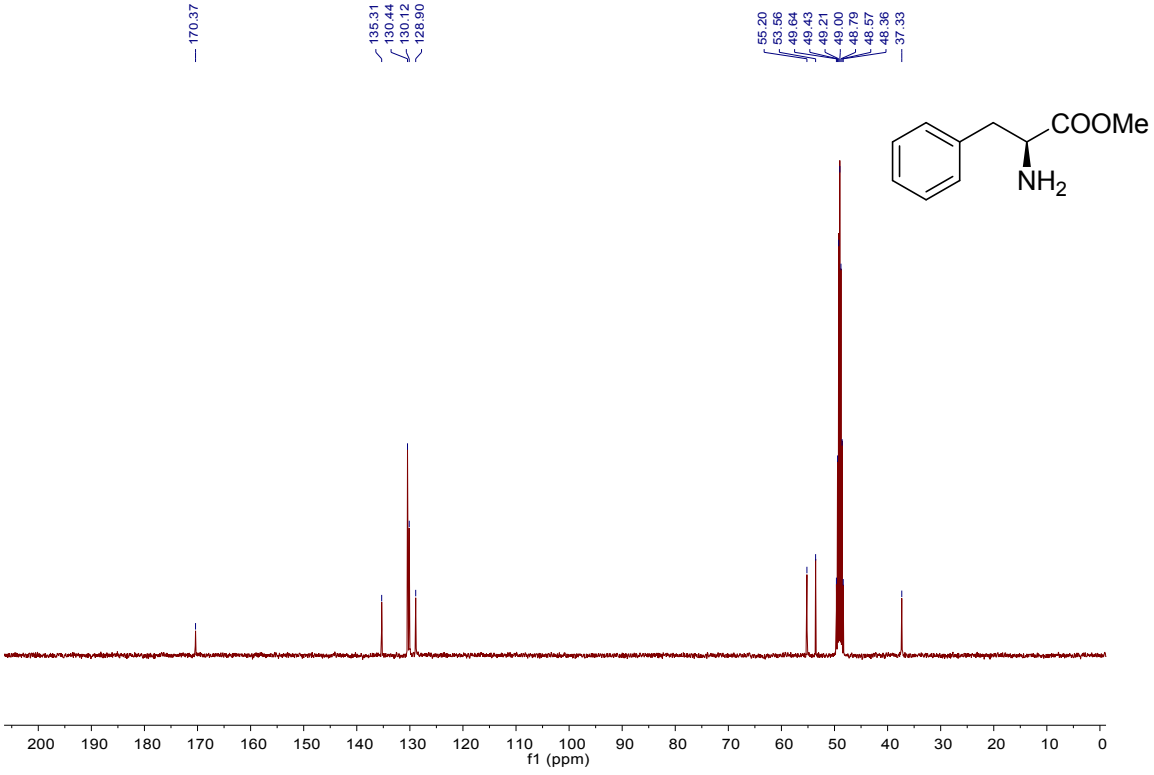


Fig. S7 Inhibition profile of the reactions of 6 amino acids with 12 selected different acids at concentration of 0.2 mM. UDP (a known UGM inhibitor) was utilized as a positive control to validate the assay.

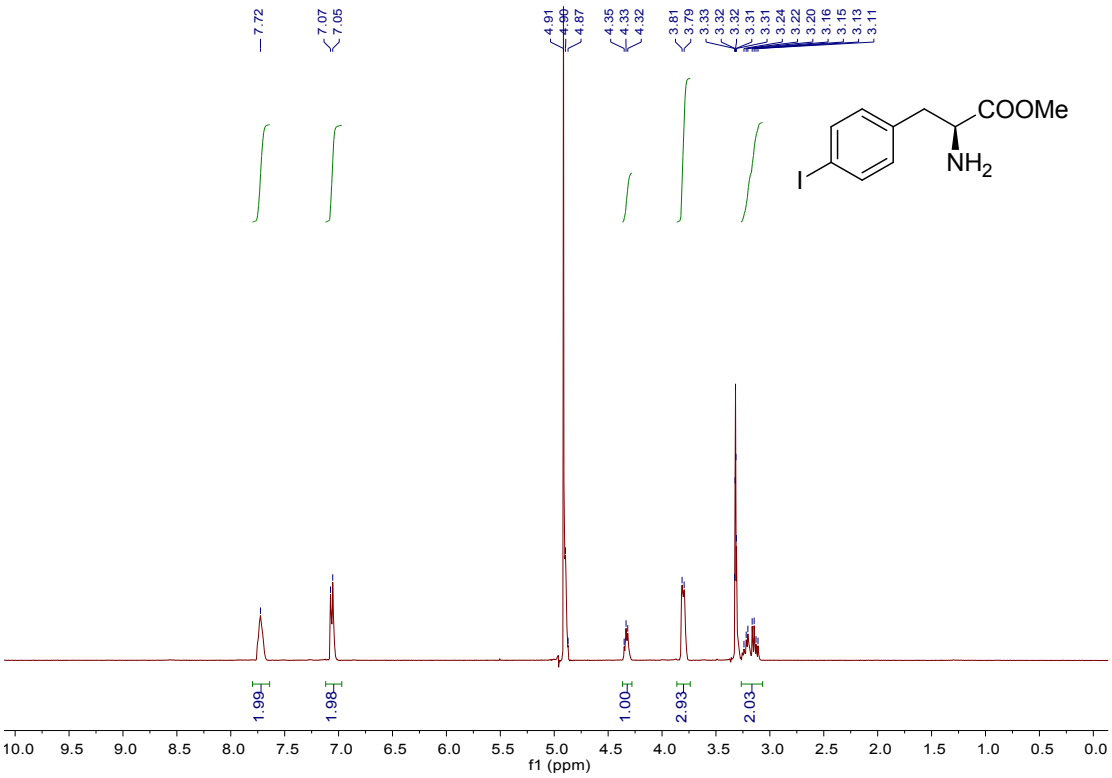
NMR spectrum and HR-MS

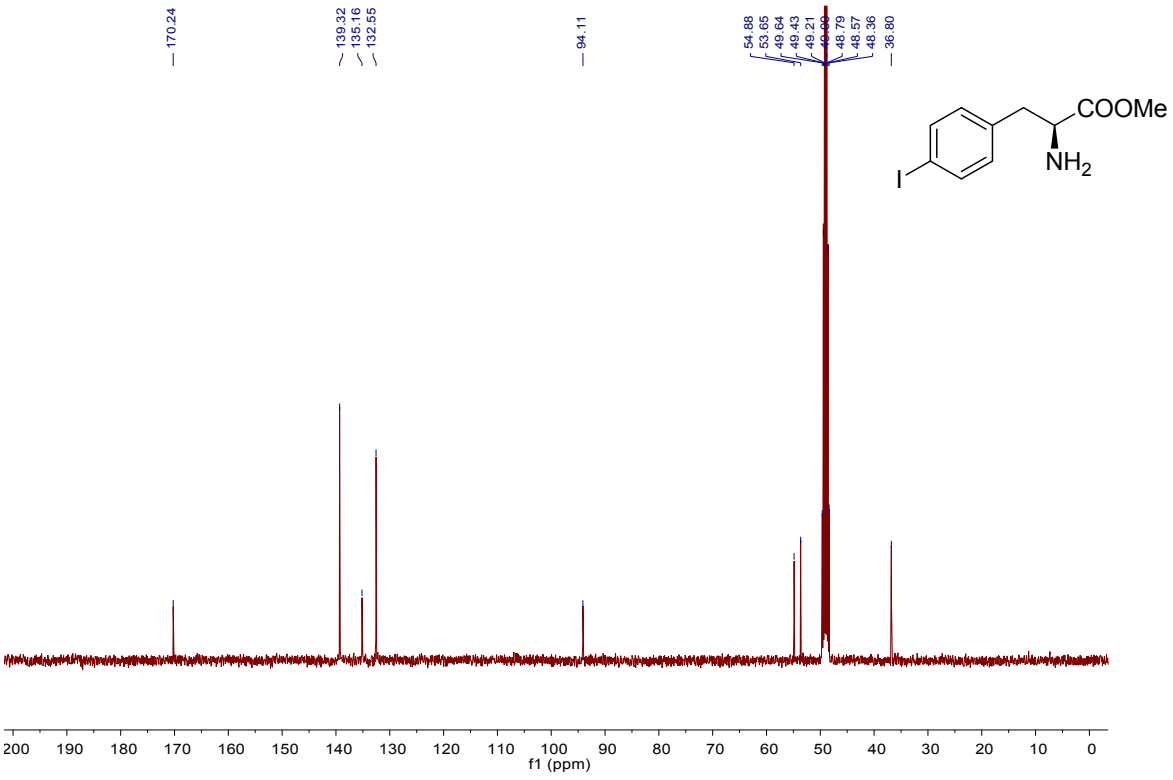
L-phenylalanine methyl ester (CD_3OD)



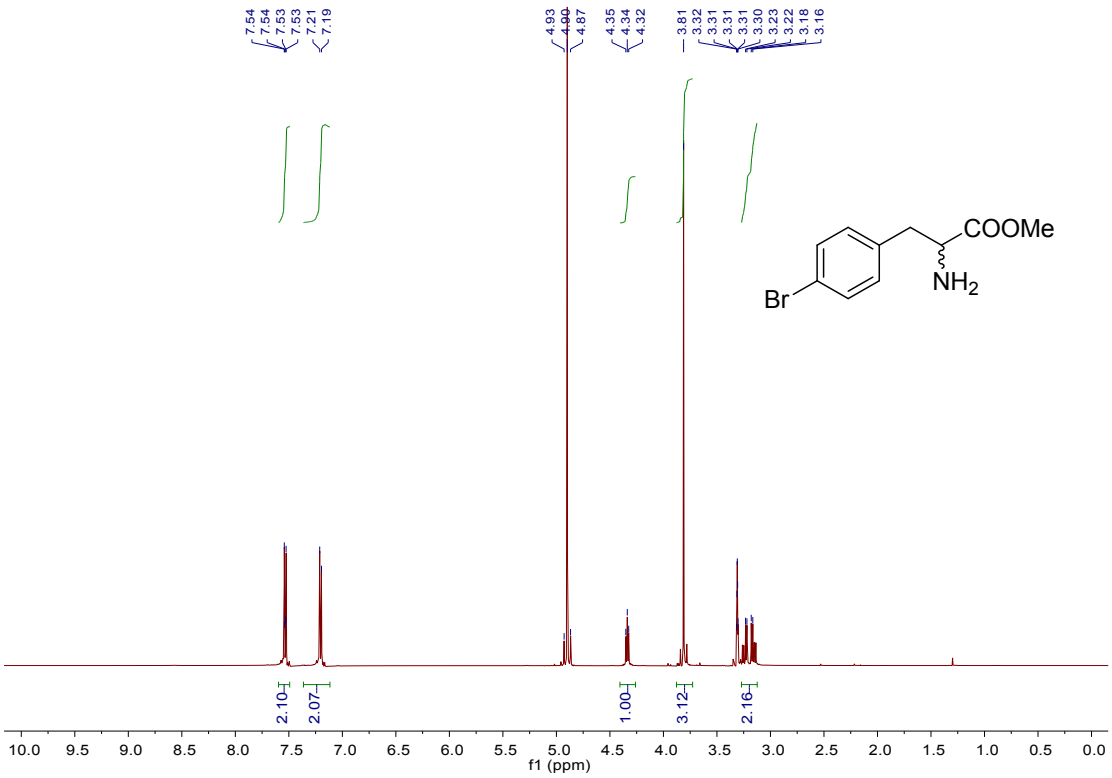


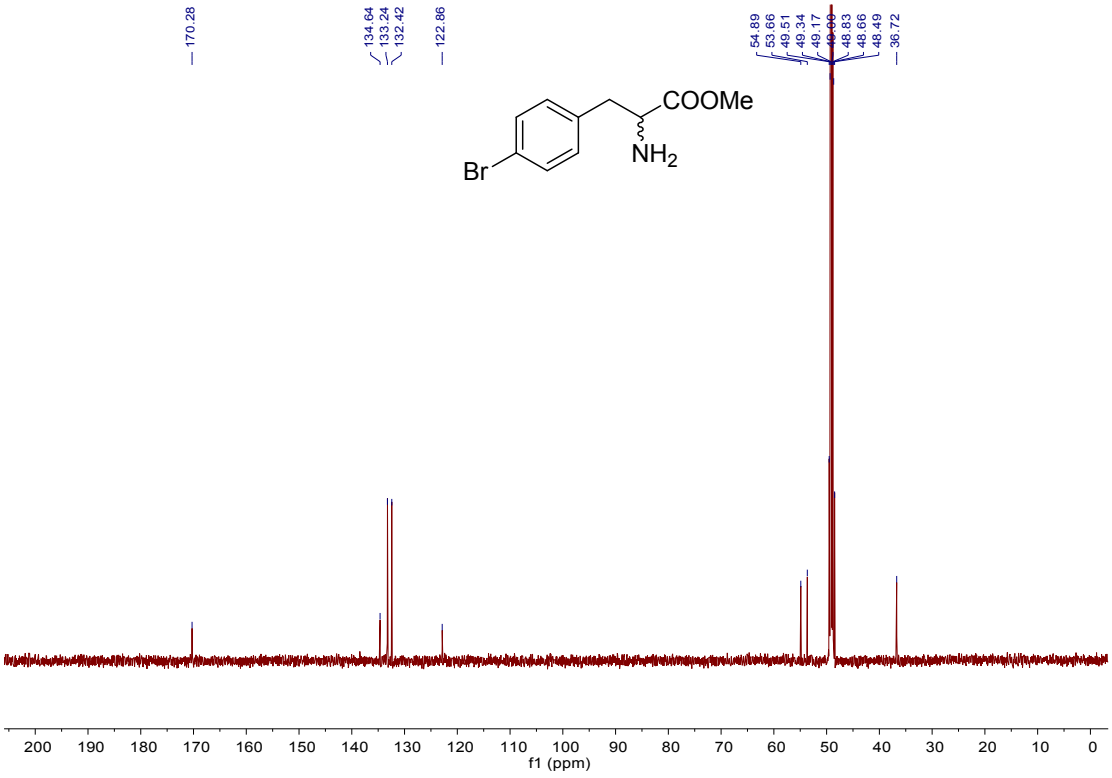
4-Iodo-L-phenylalanine methyl ester (CD_3OD)



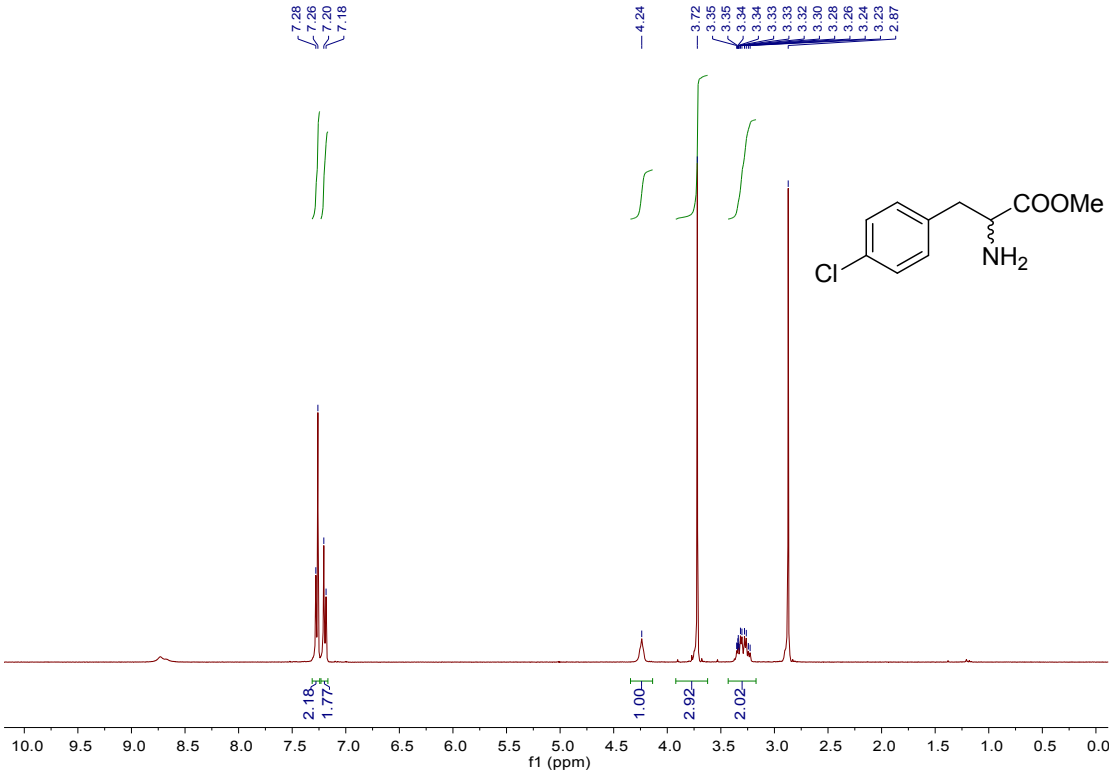


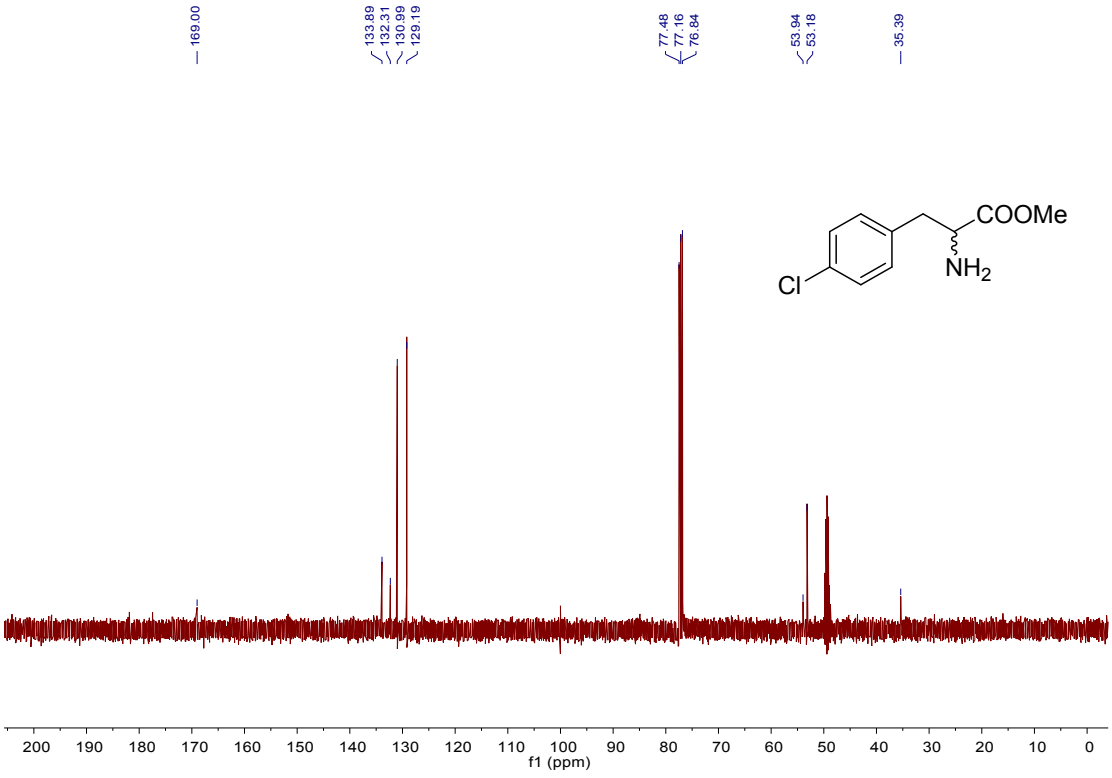
4-Bromo-DL-phenylalanine methyl ester (CD_3OD)





4-Chloro-DL-phenylalanine methyl ester ($CDCl_3$)





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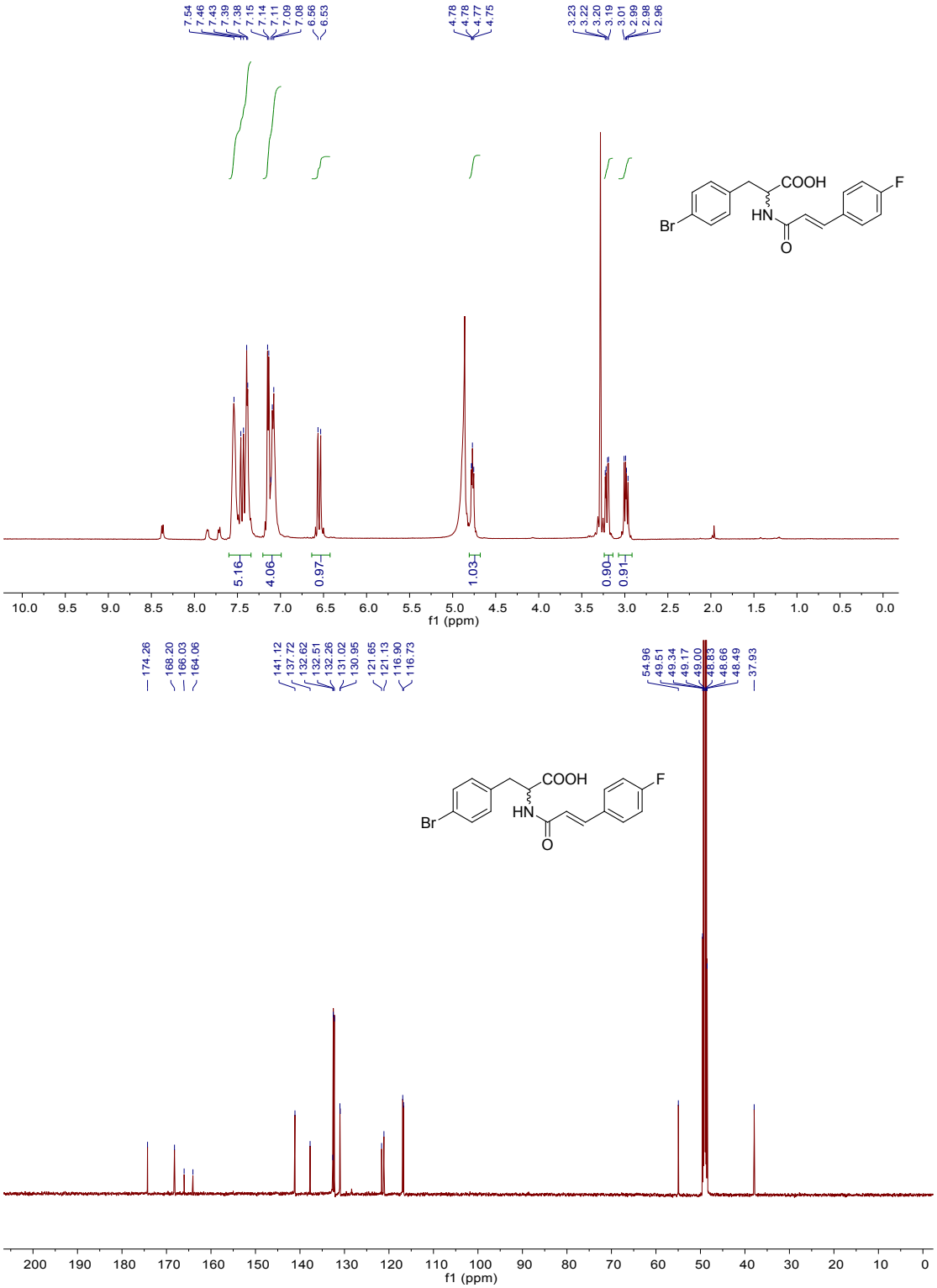
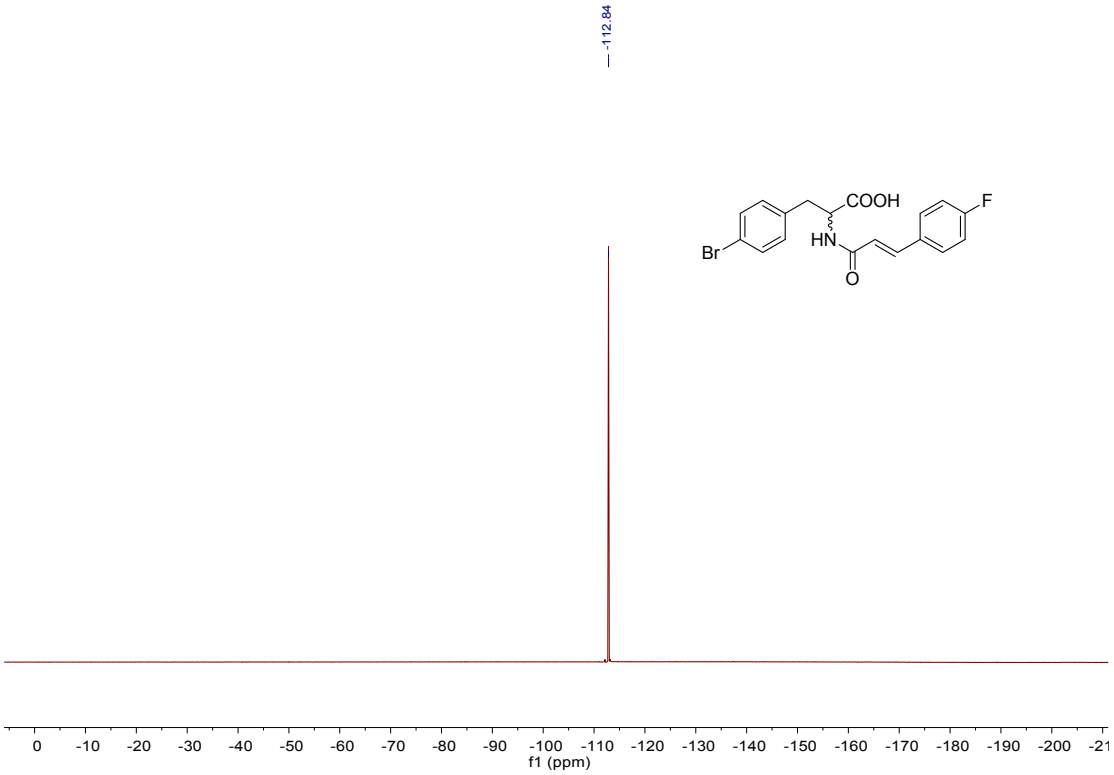


^{19}F-NMR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F
HN
Br
O
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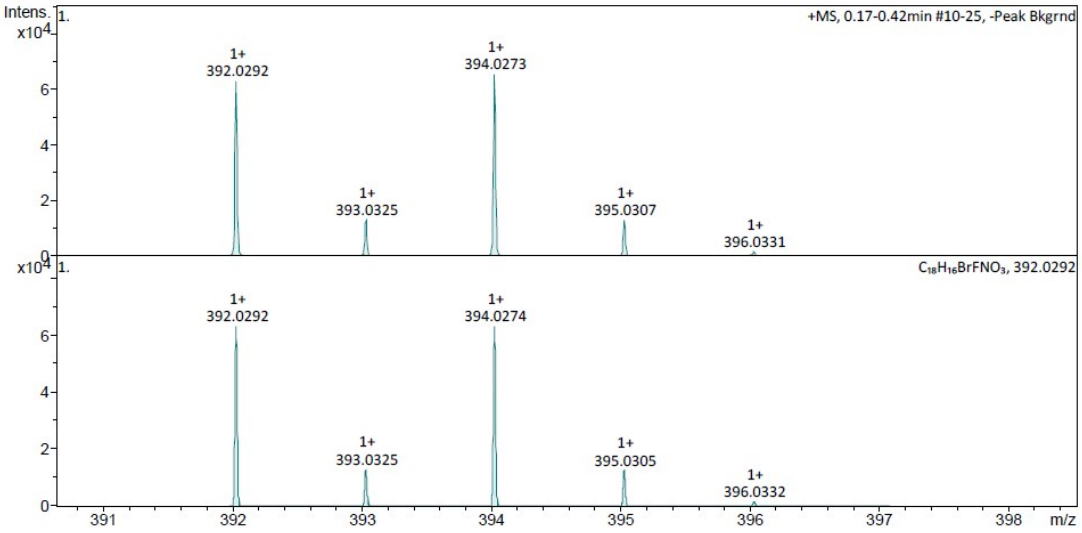
f1 (ppm)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1+ 393.0325
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1+ 395.0307
1+ 396.0331
x10^4
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1+ 392.0292
1+ 393.0325
1+ 394.0274
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1+ 396.0332
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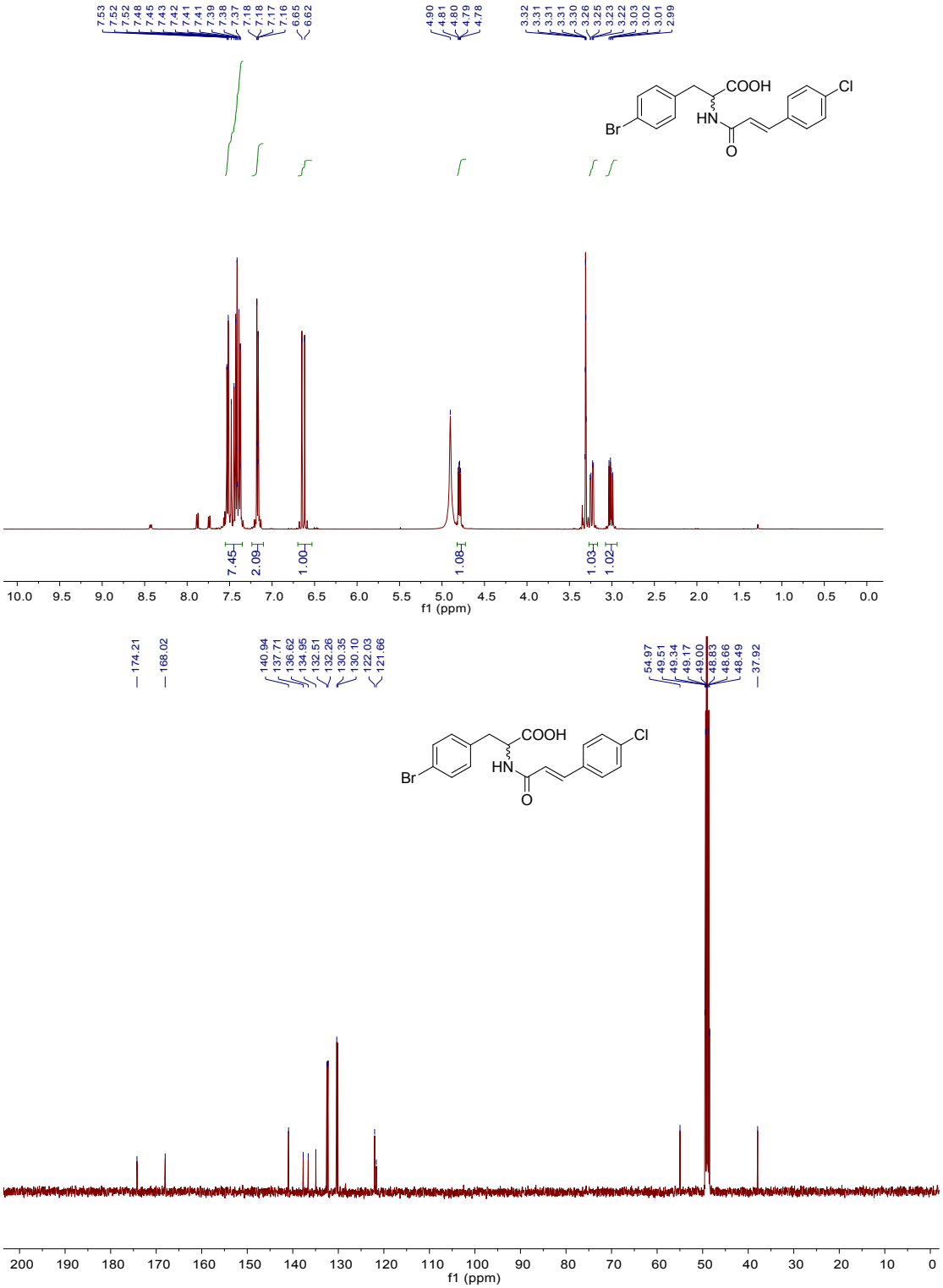
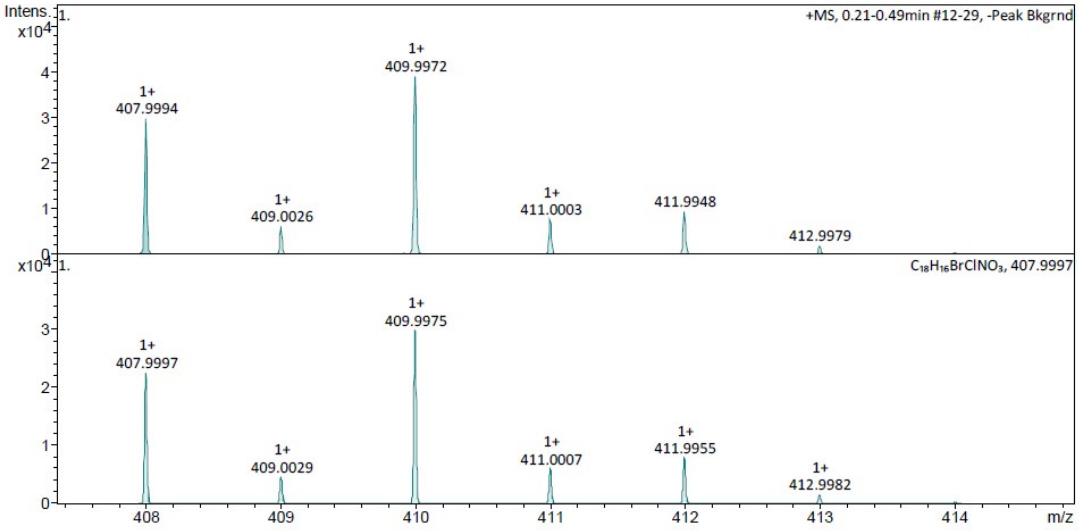
N-(4-chlorocinnamoyl)-4-Bromo-DL-phenylalanine (CD_3OD)

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1+
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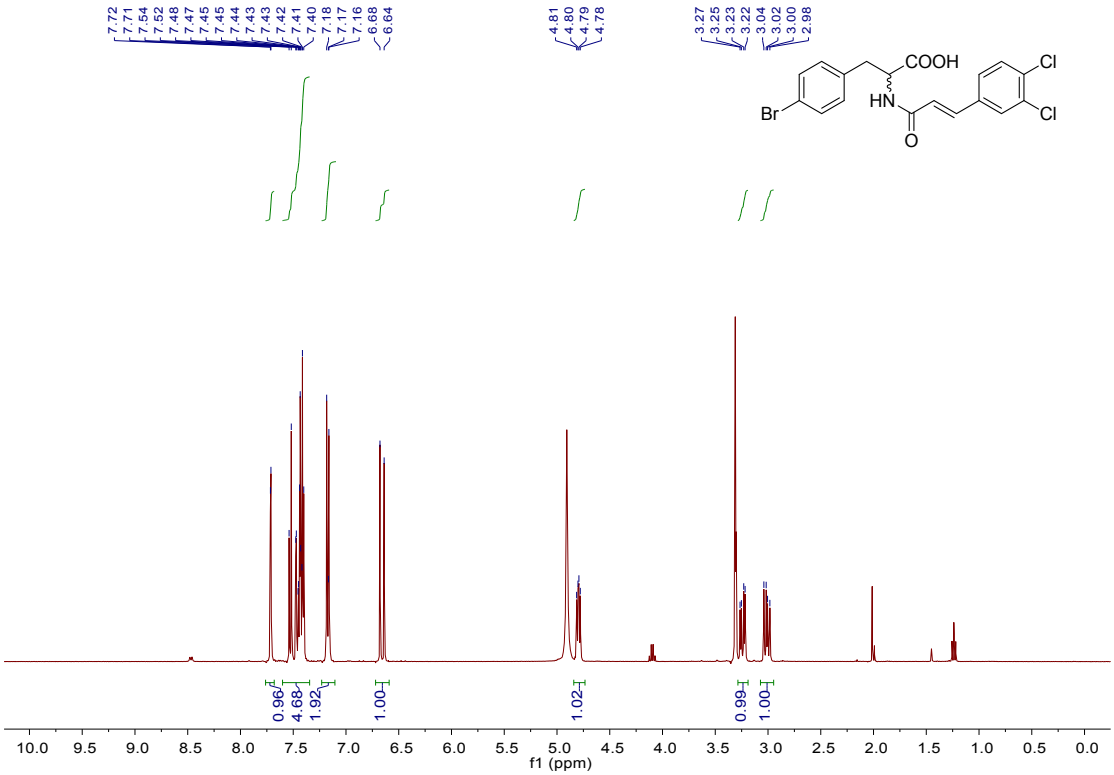
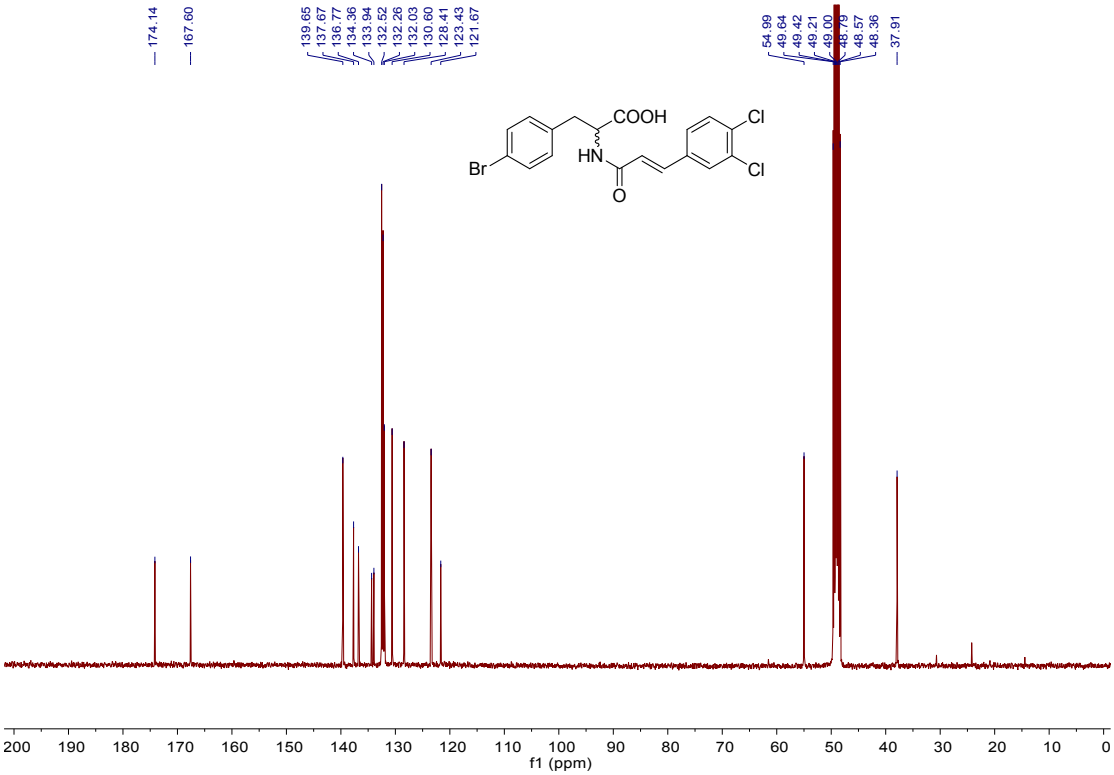
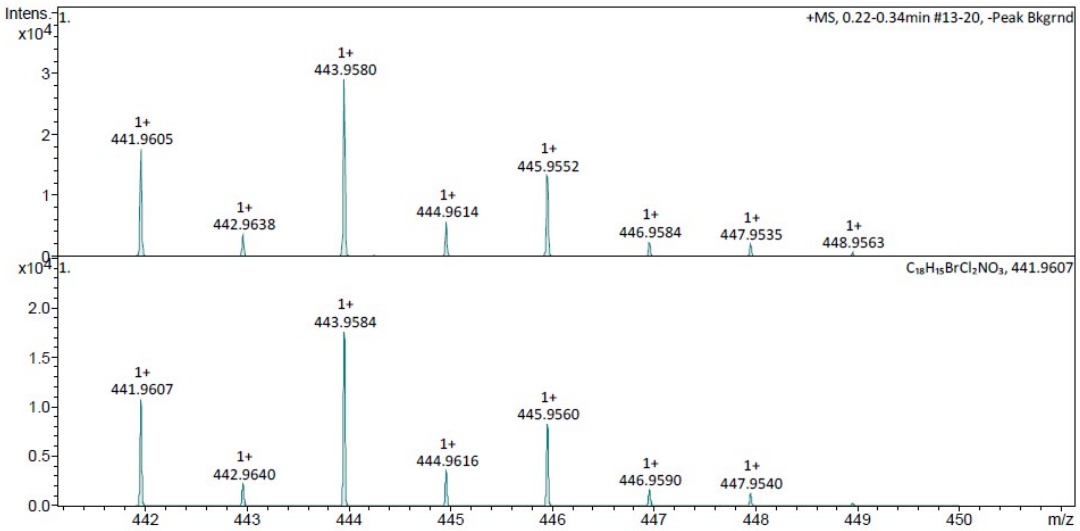
1.
C18H16BrClNO3, 407.9997
1+
407.9997
1+
409.0029
1+
409.9975
1+
411.0007
1+
411.9955
1+
412.9982
408
409
410
411
412
413
414
m/z

***N*-(3, 4-Dichlorocinnamoyl)-4-Bromo-DL-phenylalanine (CD_3OD)**



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448.9563
x10^4
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1+
444.9616
1+
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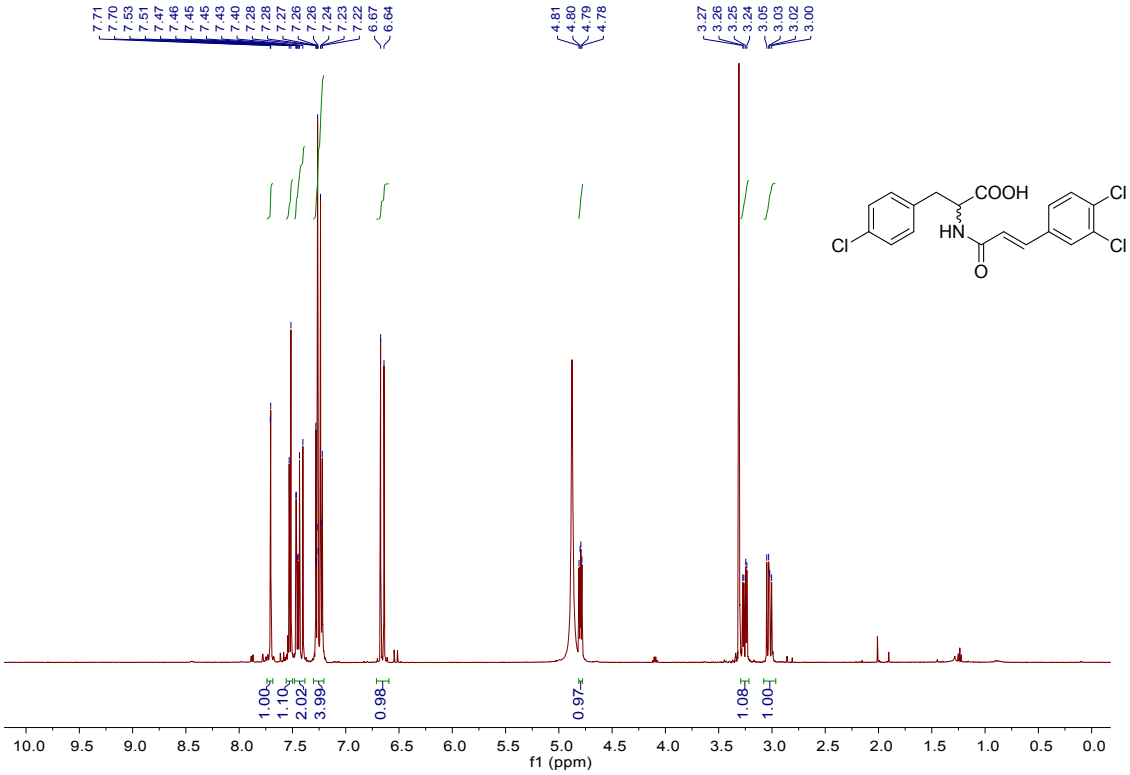
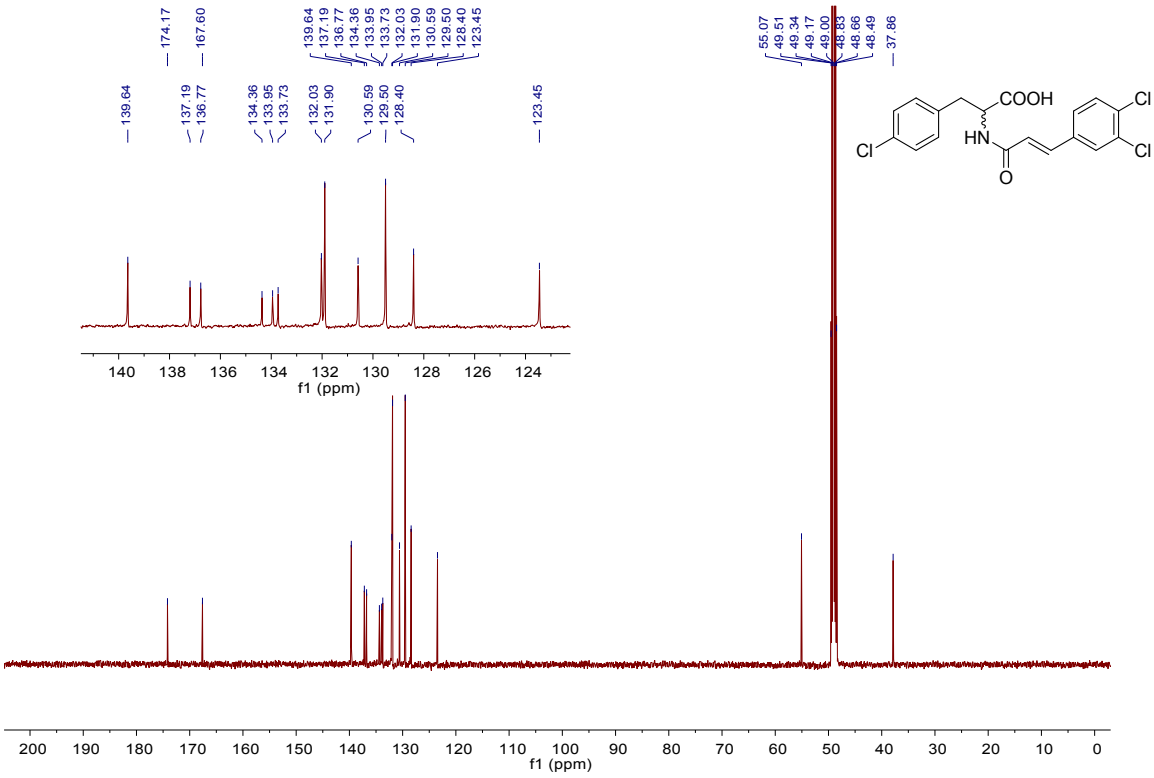
N-(3, 4-Dichlorocinnamoyl)-4-Chloro-DL-phenylalanine (CD_3OD)

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55.07
49.51
49.34
49.17
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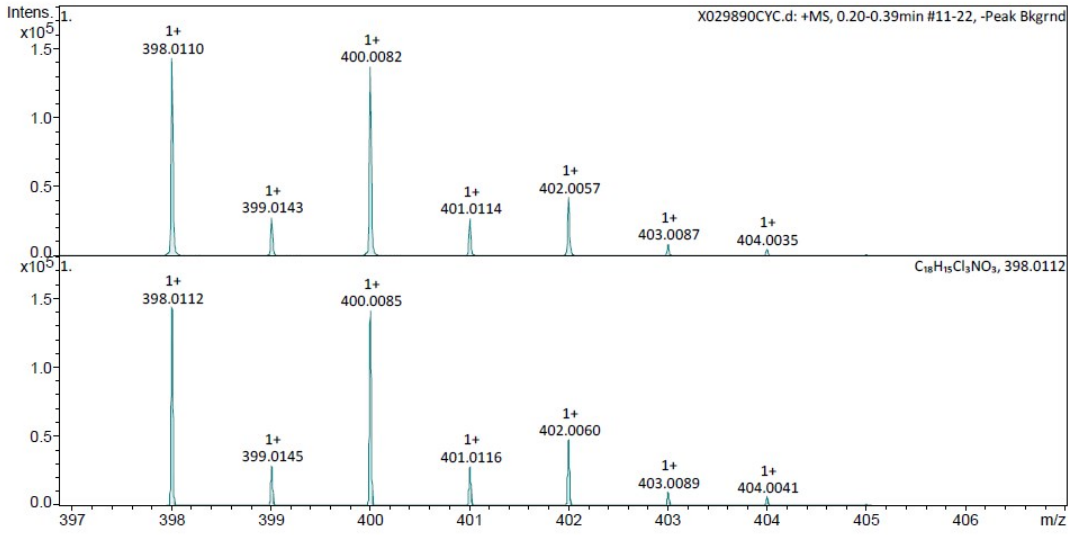
HN
O
Cl
Cl
Cl
f1 (ppm)
f1 (ppm)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404.0035
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1+
398.0112
1+
400.0085
1+
399.0145
1+
401.0116
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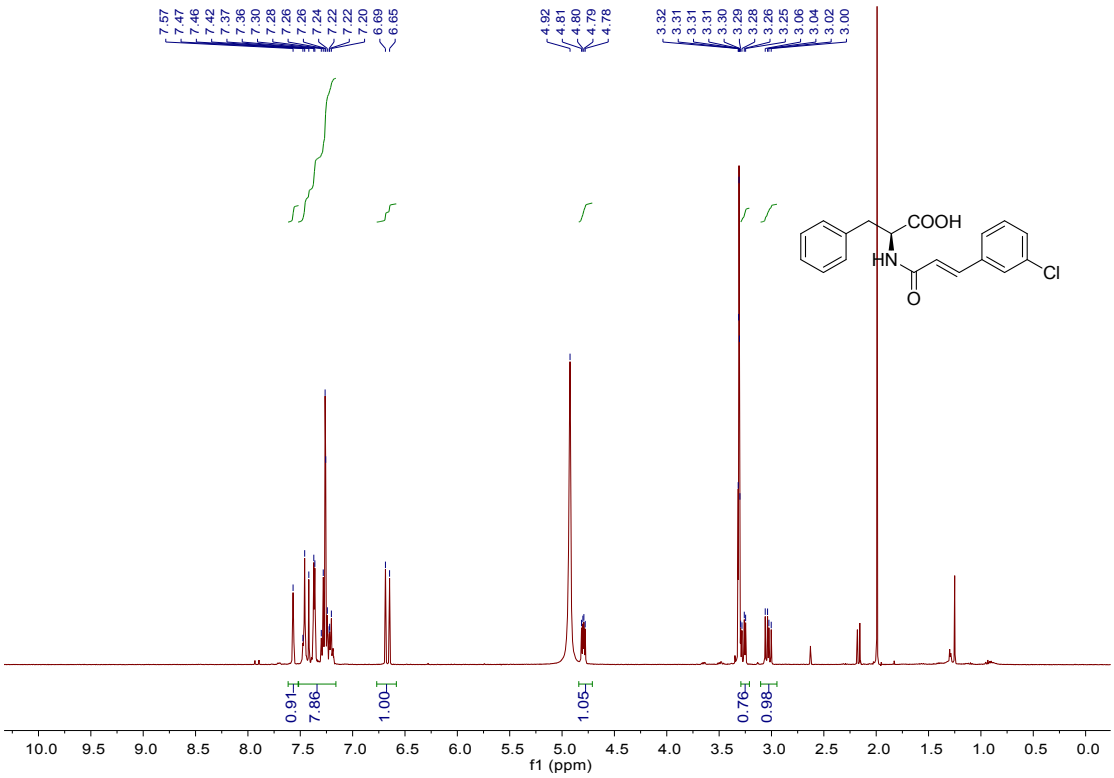
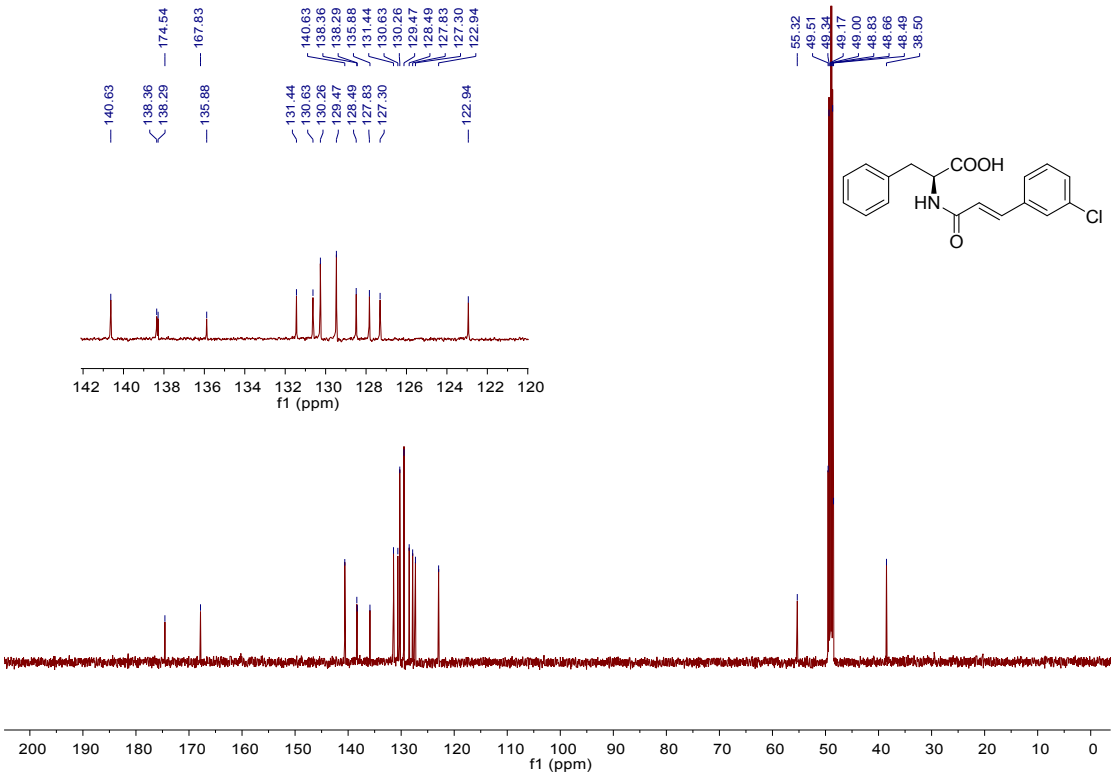
1+
404.0041
m/z

N-(3-chlorocinnamoyl)-L-phenylalanine (CD_3OD)

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167.83
55.32
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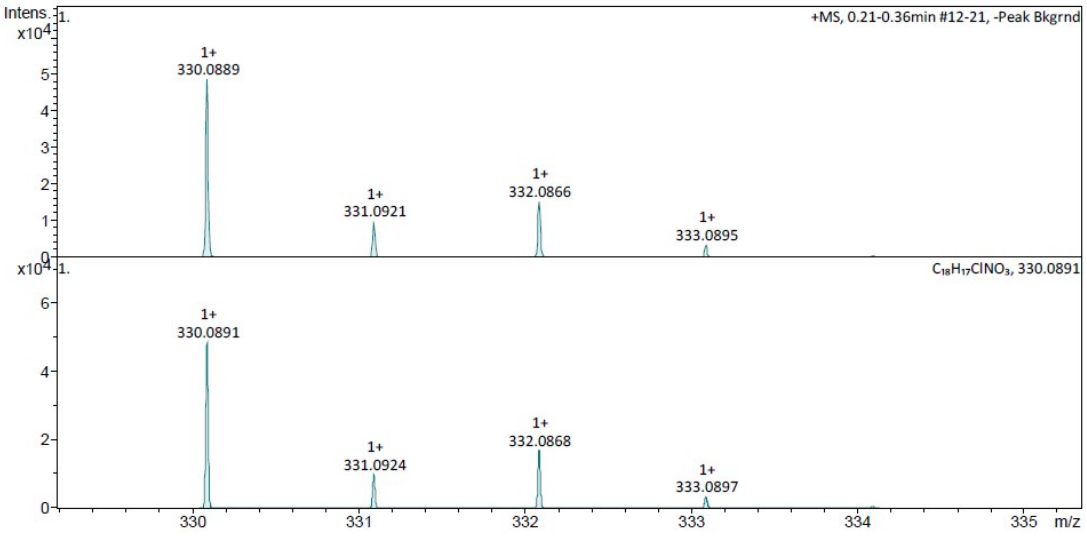
HN
O
Cl
f1 (ppm)
f1 (ppm)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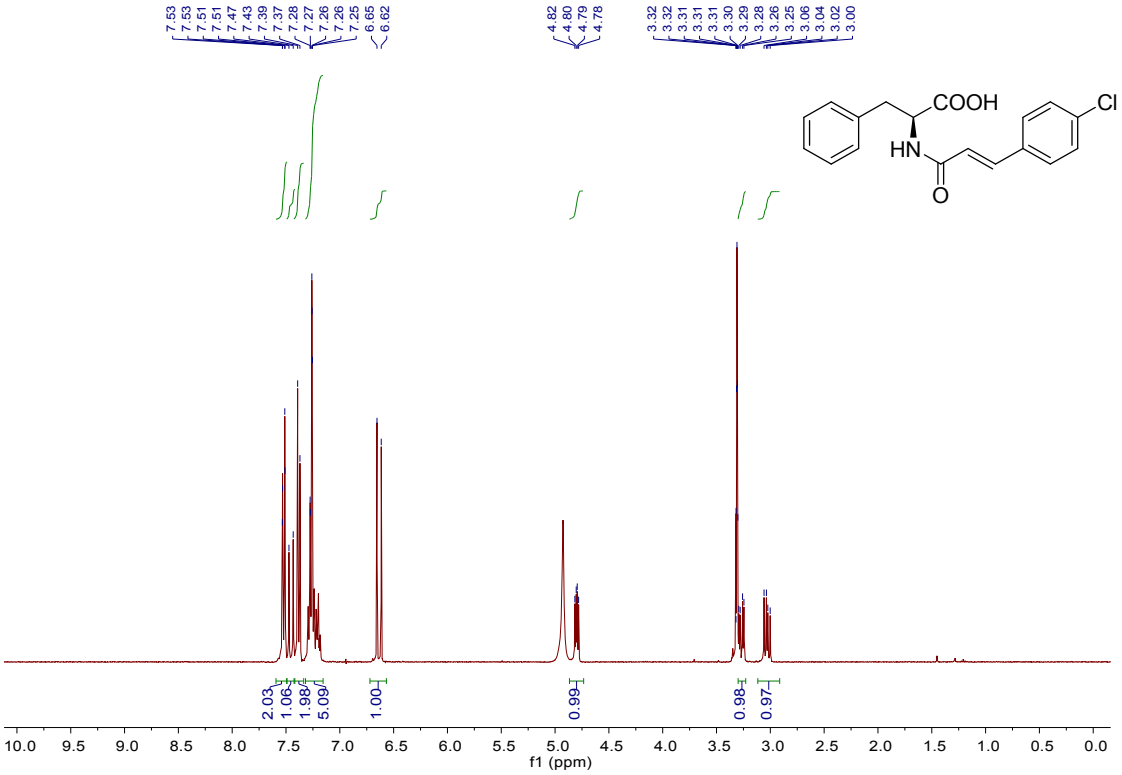
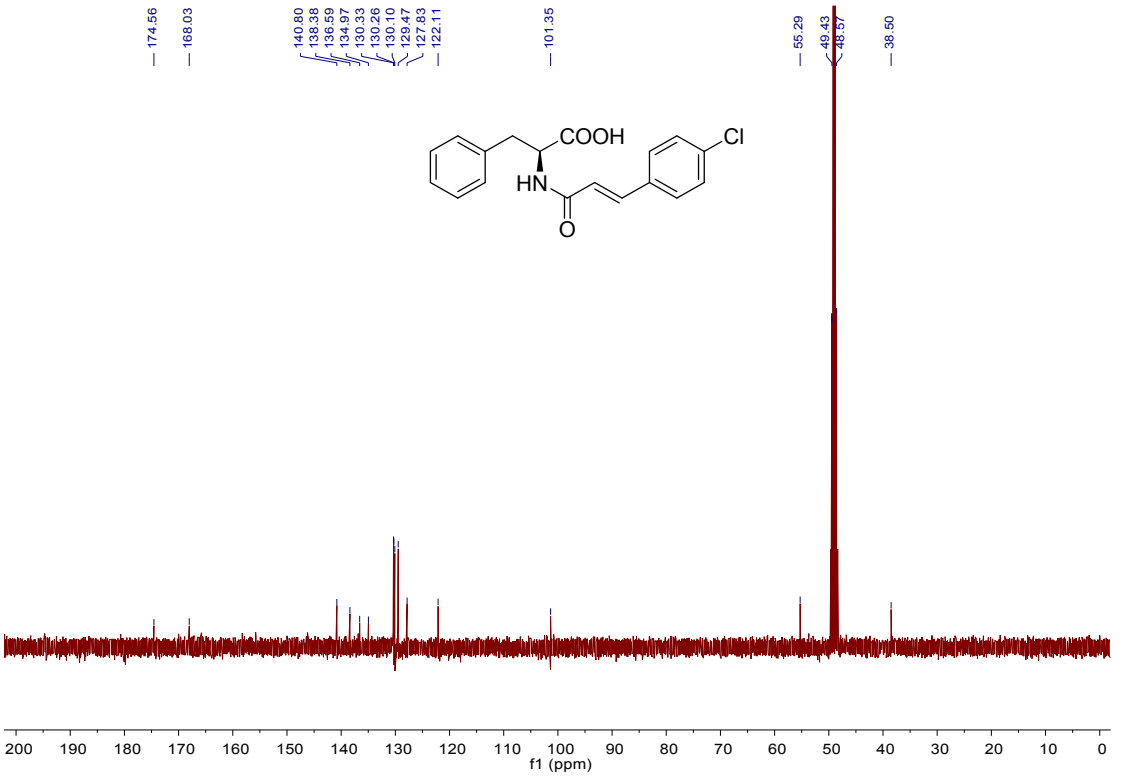
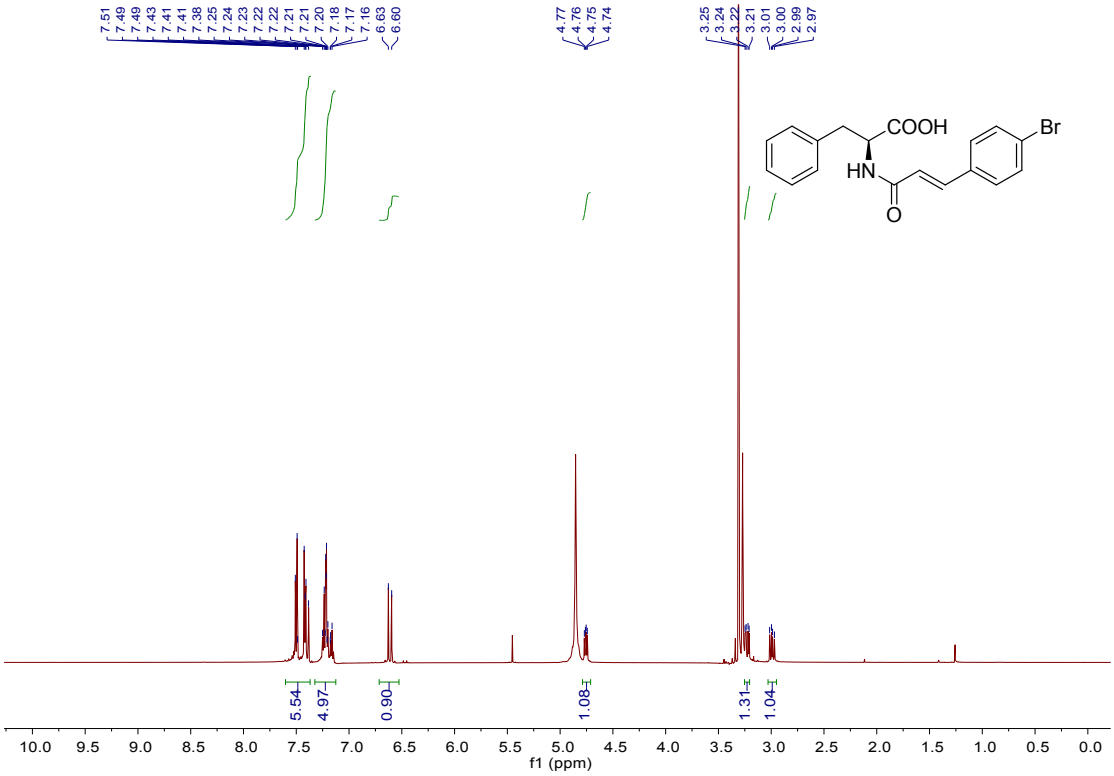
m/z

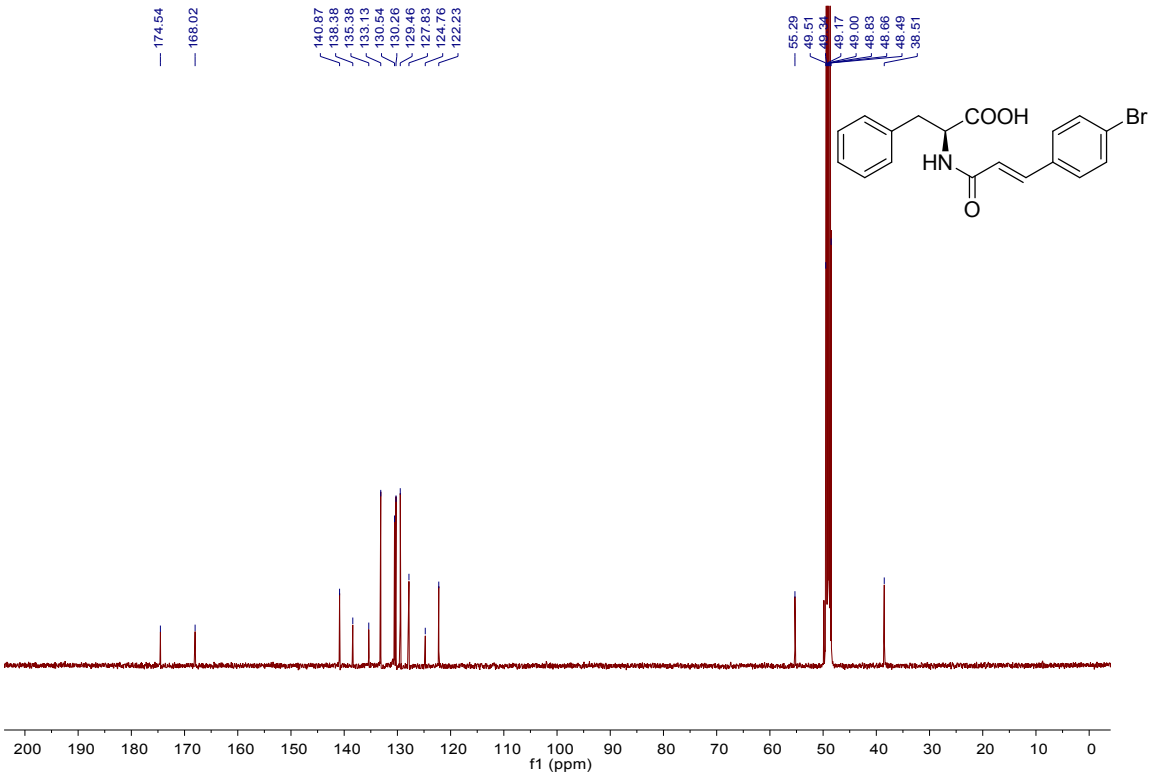
N-(4-Chlorocinnamoyl)-L-phenylalanine (CD_3OD)





N-(4-Bromocinnamoyl)-L-phenylalanine (CD_3OD)

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55.29
49.51
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49.17
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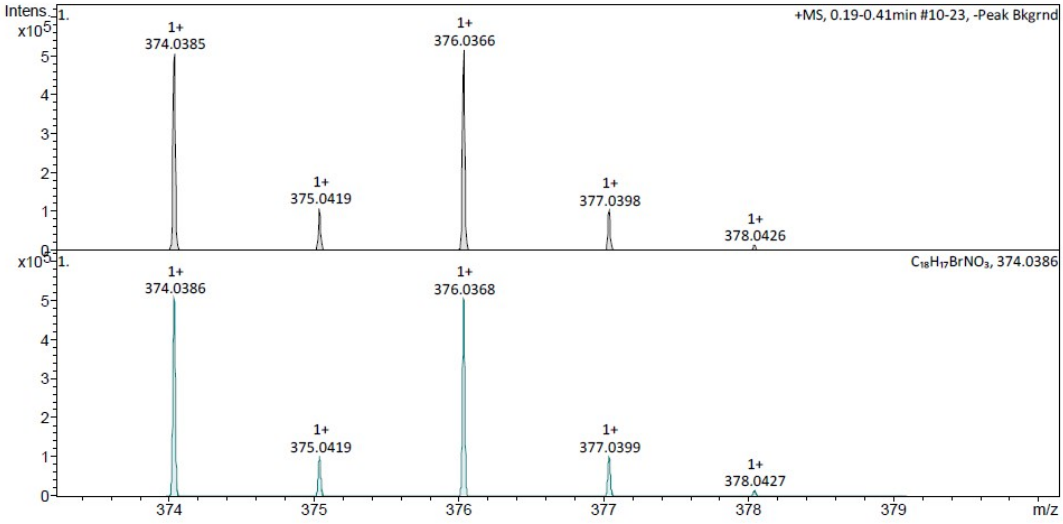
48.66
48.49
38.51
COOH
HN
O
Br
f1 (ppm)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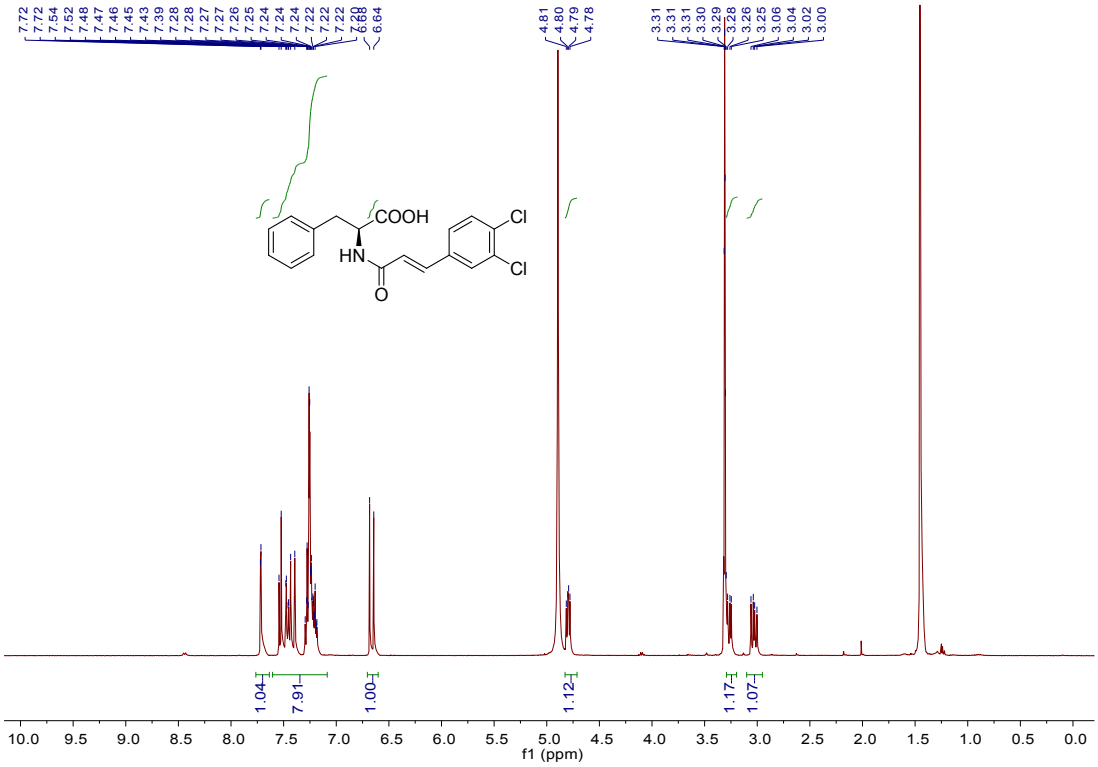
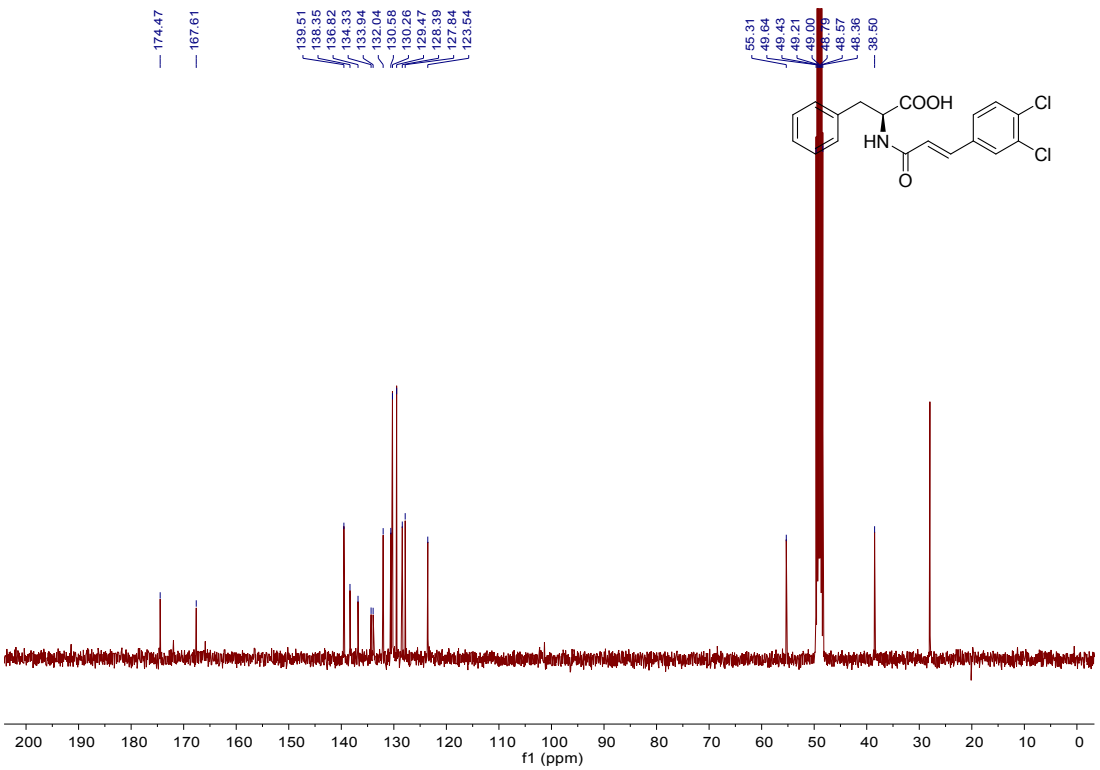
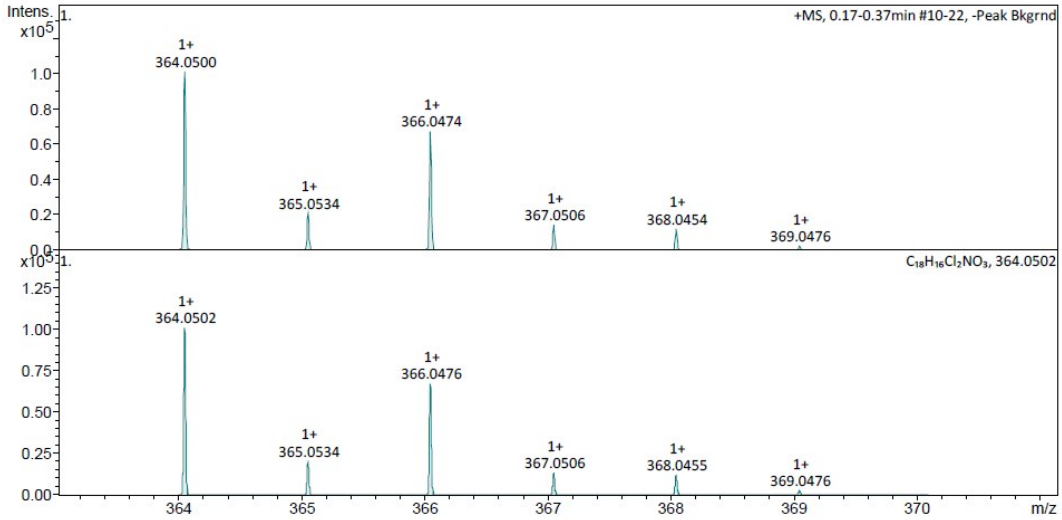
1+ 375.0419
1+ 376.0366
1+ 377.0398
1+ 378.0426
C18H17BrNO3, 374.0386
1+ 374.0386
1+ 375.0419
1+ 376.0368
1+ 377.0399
1+ 378.0427
m/z

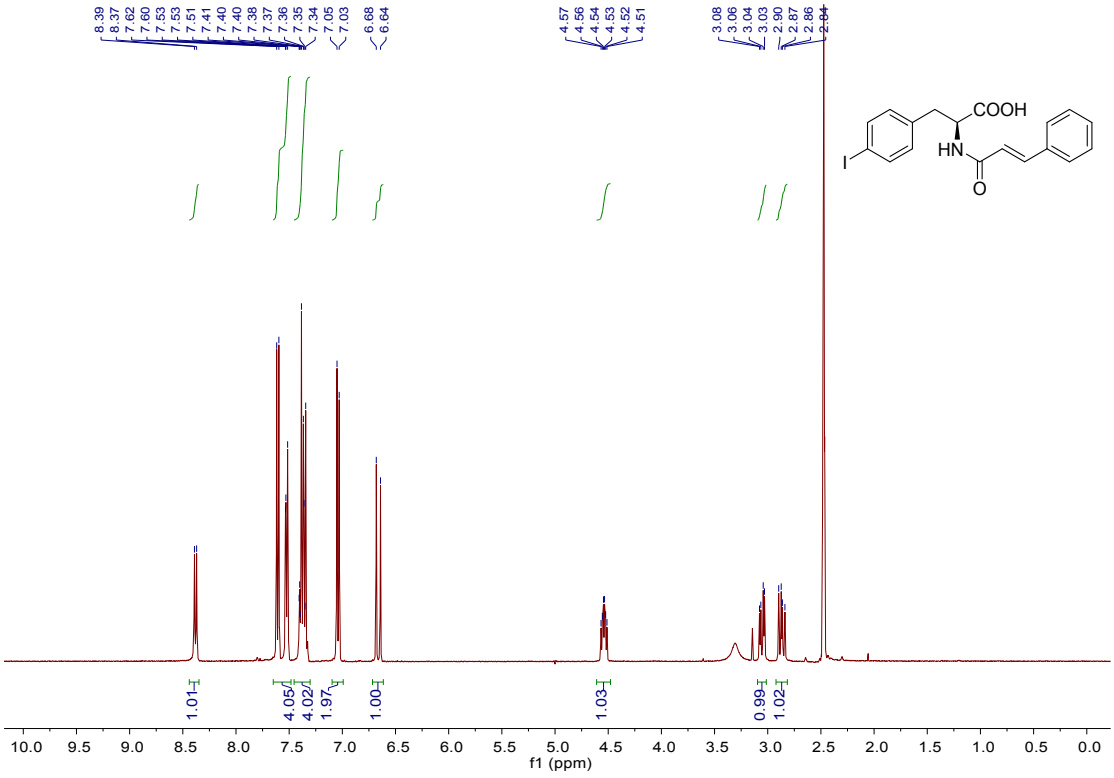
N-(3, 4-Dichlorocinnamoyl)-L-phenylalanine (CD_3OD)

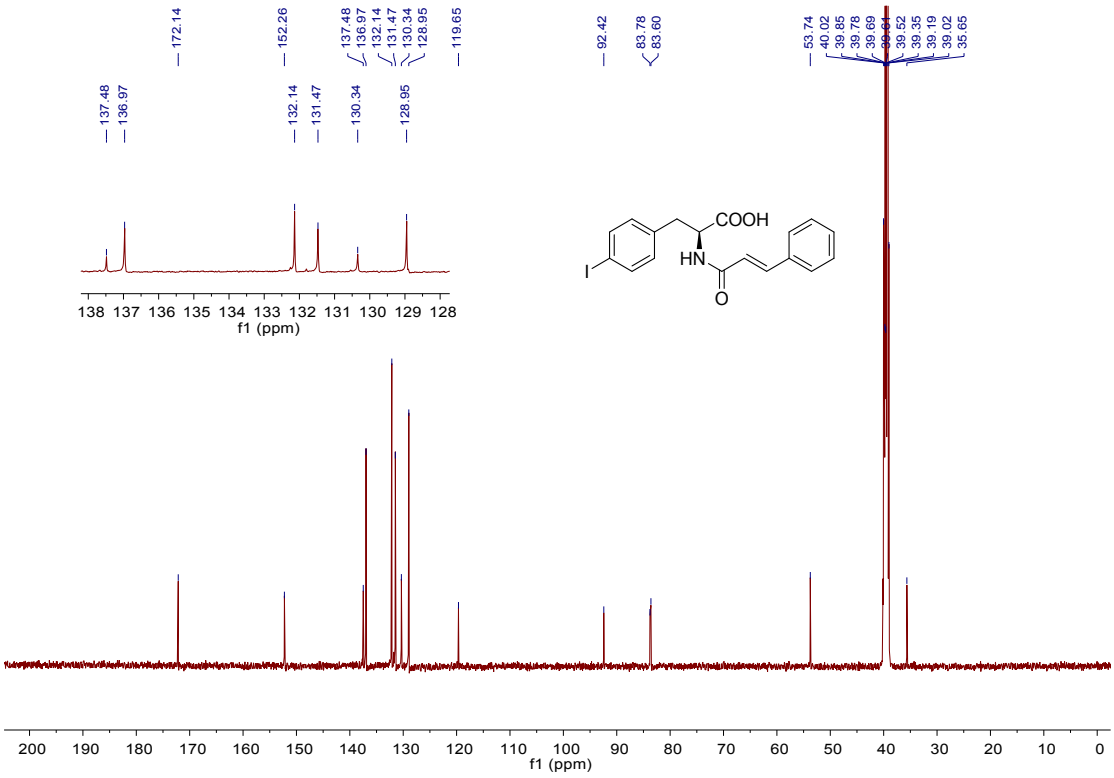


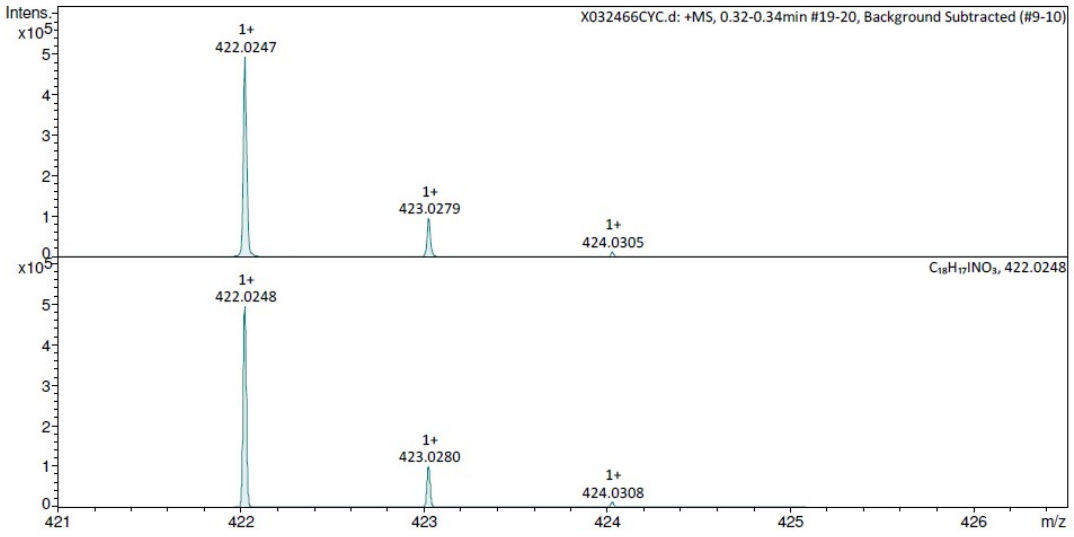

174.47
167.61
139.51
138.35
136.82
134.33
133.94
132.04
130.58
130.26
129.47
128.39
127.84
123.54
55.31
49.64
49.43
49.21
49.00
48.79
48.57
48.36
38.50
COOH
HN
O
Cl
Cl
f1 (ppm)


Intens.
x10^5
1.
+MS, 0.17-0.37min #10-22, -Peak Bkgrnd
1+ 364.0500
1+ 365.0534
1+ 366.0474
1+ 367.0506
1+ 368.0454
1+ 369.0476
C18H16Cl2NO3, 364.0502
1+ 364.0502
1+ 365.0534
1+ 366.0476
1+ 367.0506
1+ 368.0455
1+ 369.0476
m/z

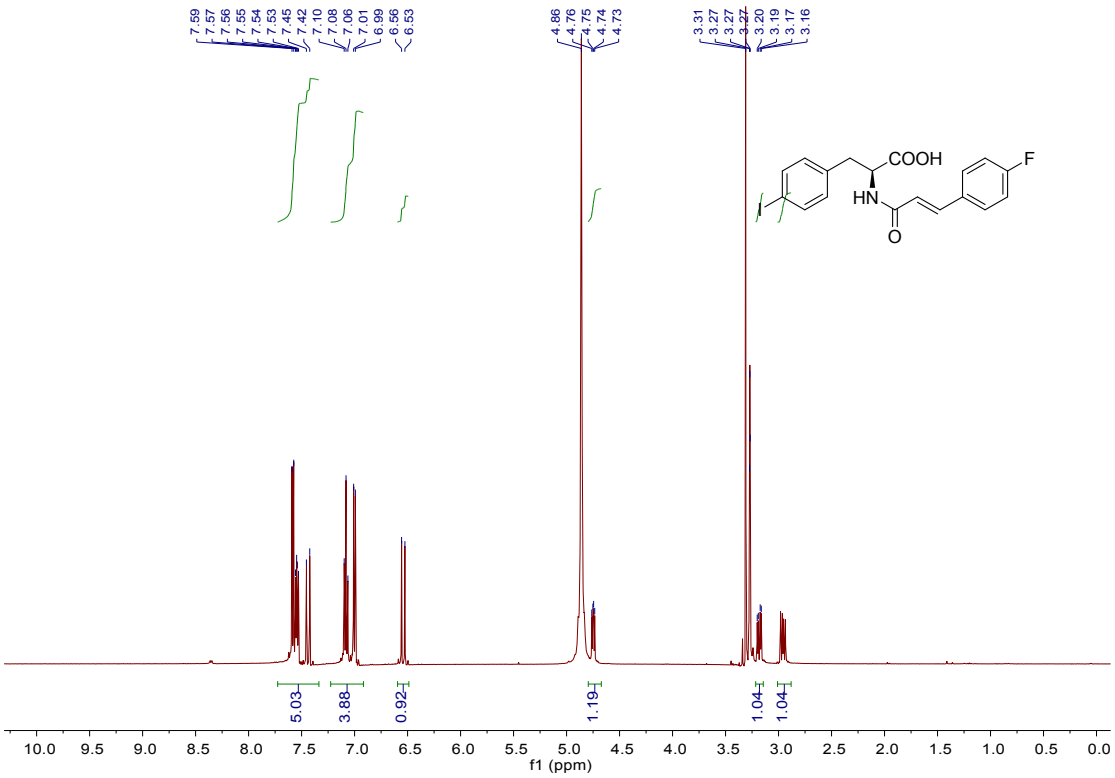
N-cinnamoyl-4-Iodo-L-phenylalanine (DMSO-*d6*)

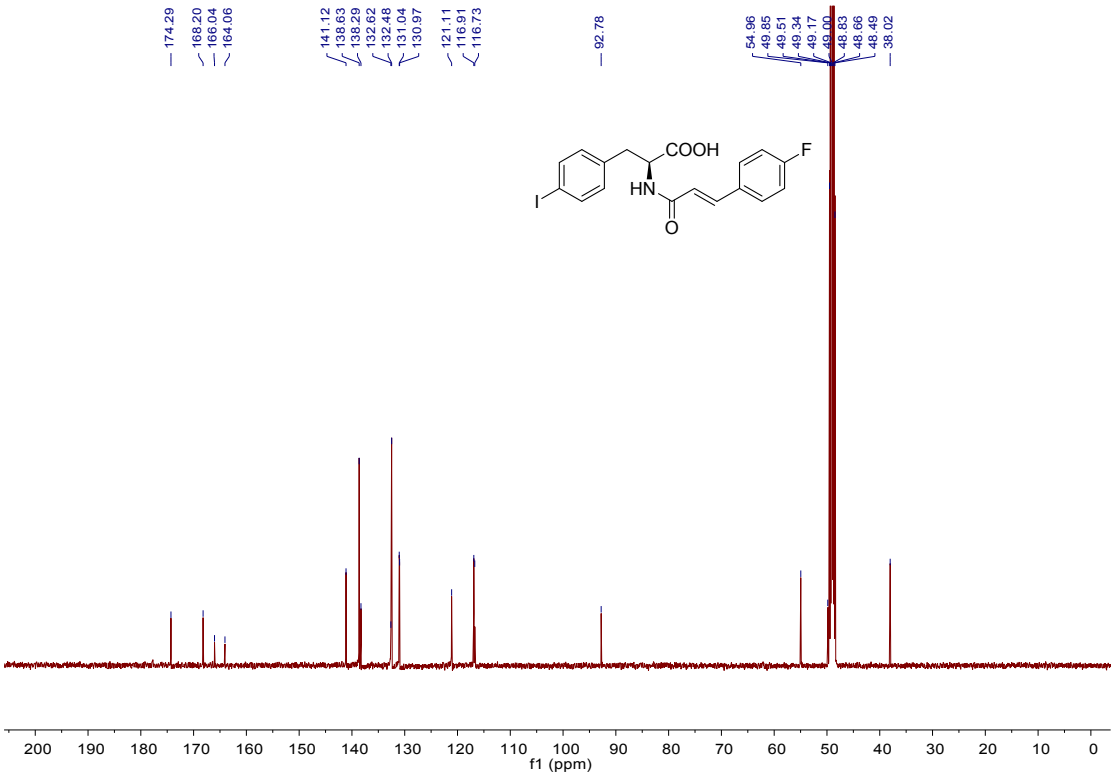


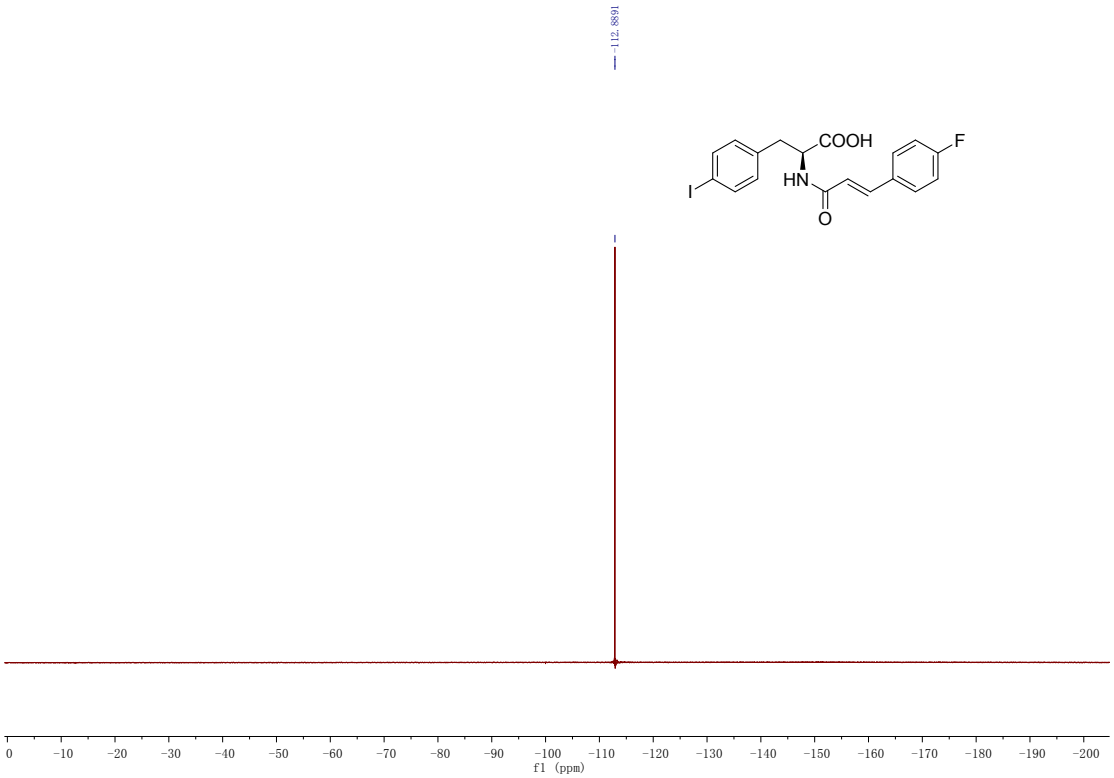

172.14
152.26
137.48
136.97
132.14
131.47
130.34
128.95
119.65
92.42
83.78
83.60
53.74
40.02
39.85
39.78
39.69
39.52
39.35
39.19
39.02
35.65
137.48
136.97
132.14
131.47
130.34
128.95
138 137 136 135 134 133 132 131 130 129 128
f1 (ppm)
COOH
HN
O
I
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

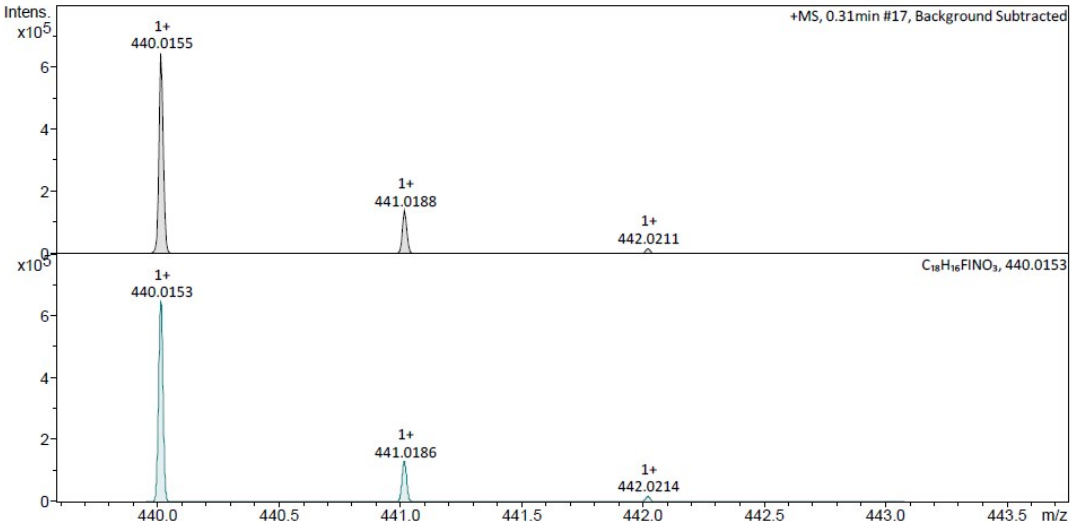

Intens.
x10^5
X032466CYC.d: +MS, 0.32-0.34min #19-20, Background Subtracted (#9-10)
1+
422.0247
1+
423.0279
1+
424.0305
x10^5
C18H17INO3, 422.0248
1+
422.0248
1+
423.0280
1+
424.0308
421
422
423
424
425
426
m/z

N-(4-fluorocinnamoyl)-4-iodo-L-phenylalanine (CD_3OD)

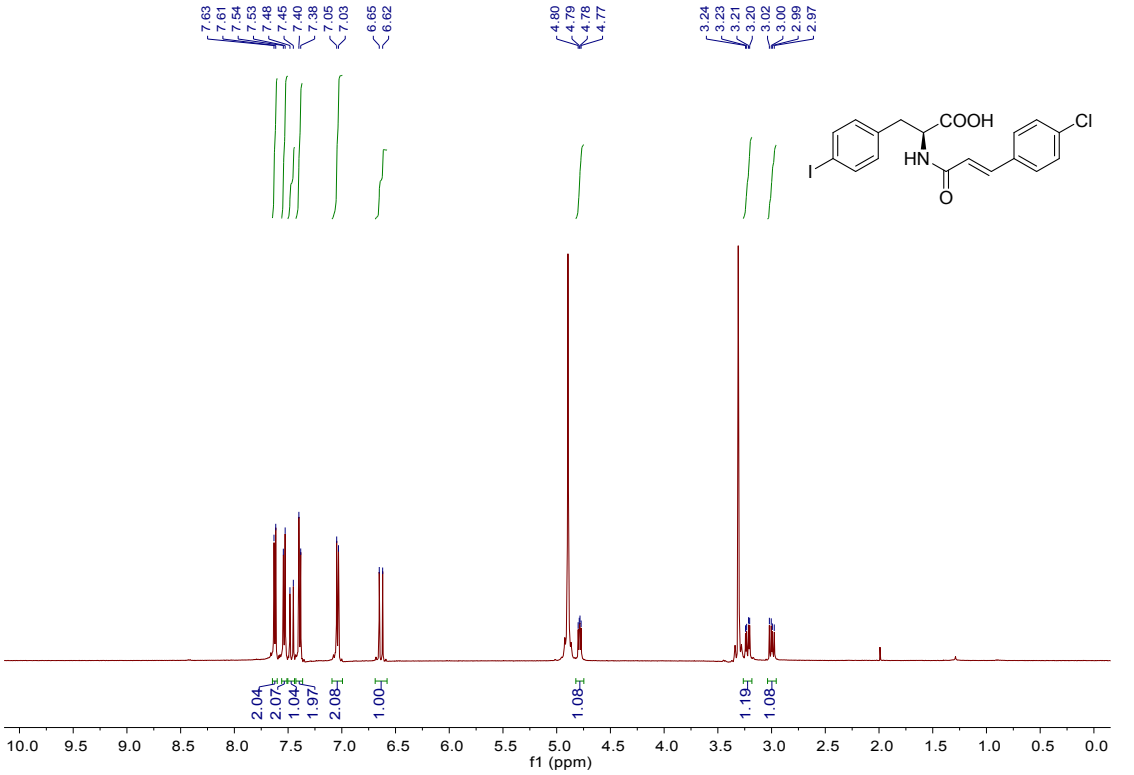


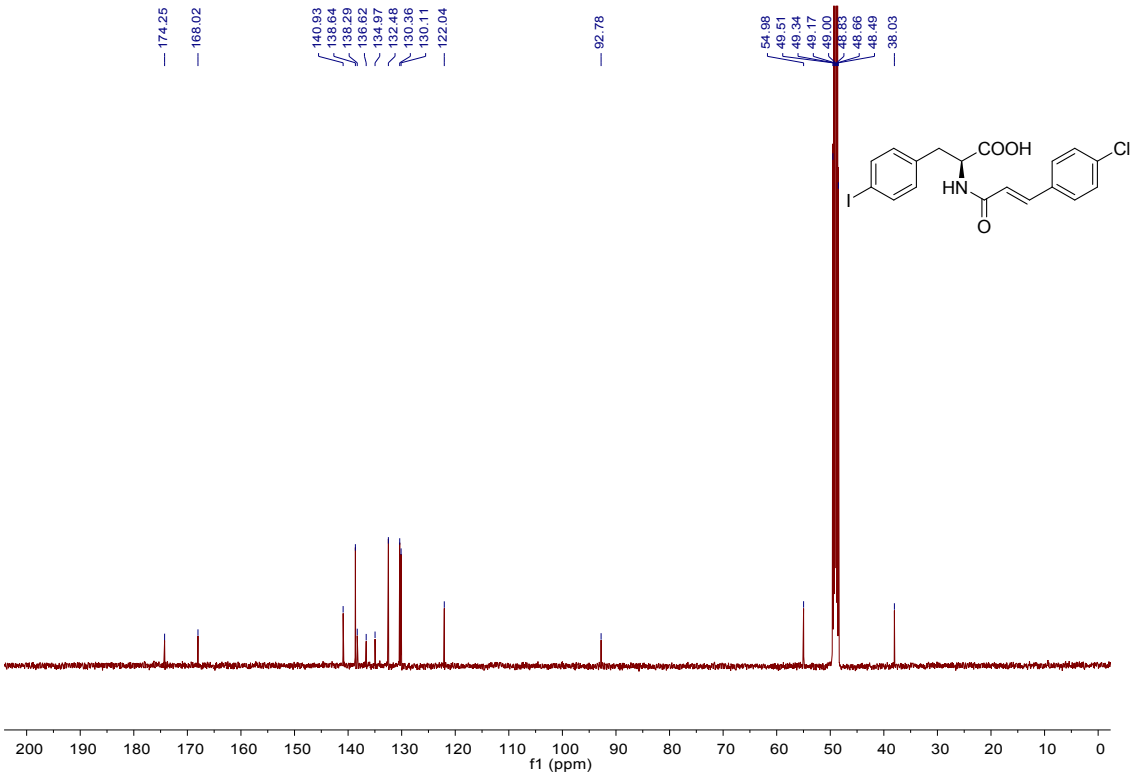


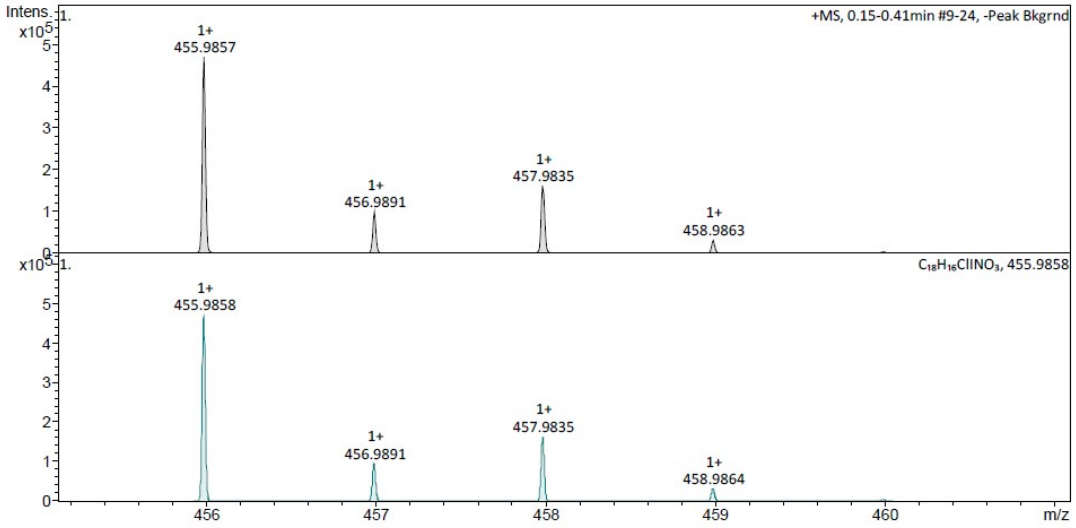

-112.8891
COOH
F
HN
I
O
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
−110
−120
−130
−140
−150
−160
−170
−180
−190
−200
f1 (ppm)


Intens.
x10^5
+MS, 0.31min #17, Background Subtracted
1+
440.0155
1+
441.0188
1+
442.0211
C18H16FINO3, 440.0153
1+
440.0153
1+
441.0186
1+
442.0214
440.0
440.5
441.0
441.5
442.0
442.5
443.0
443.5
m/z

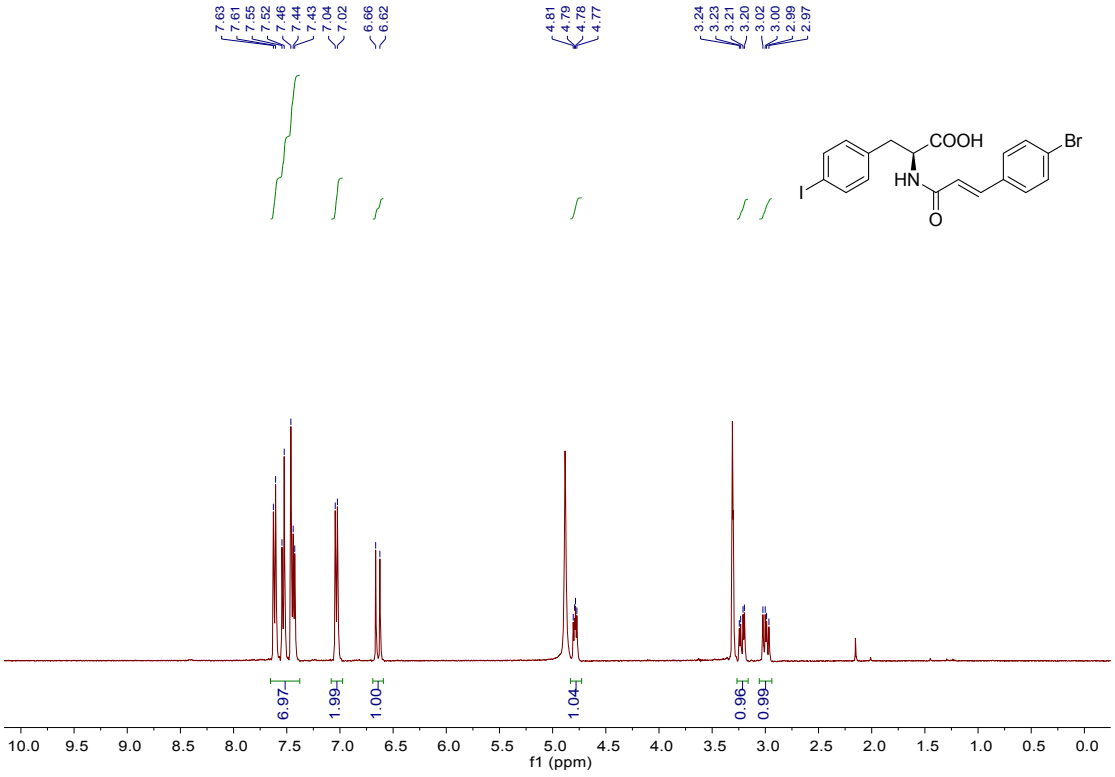
N-(4-Chlorocinnamoyl)-4-iodo-L-phenylalanine (CD_3OD)

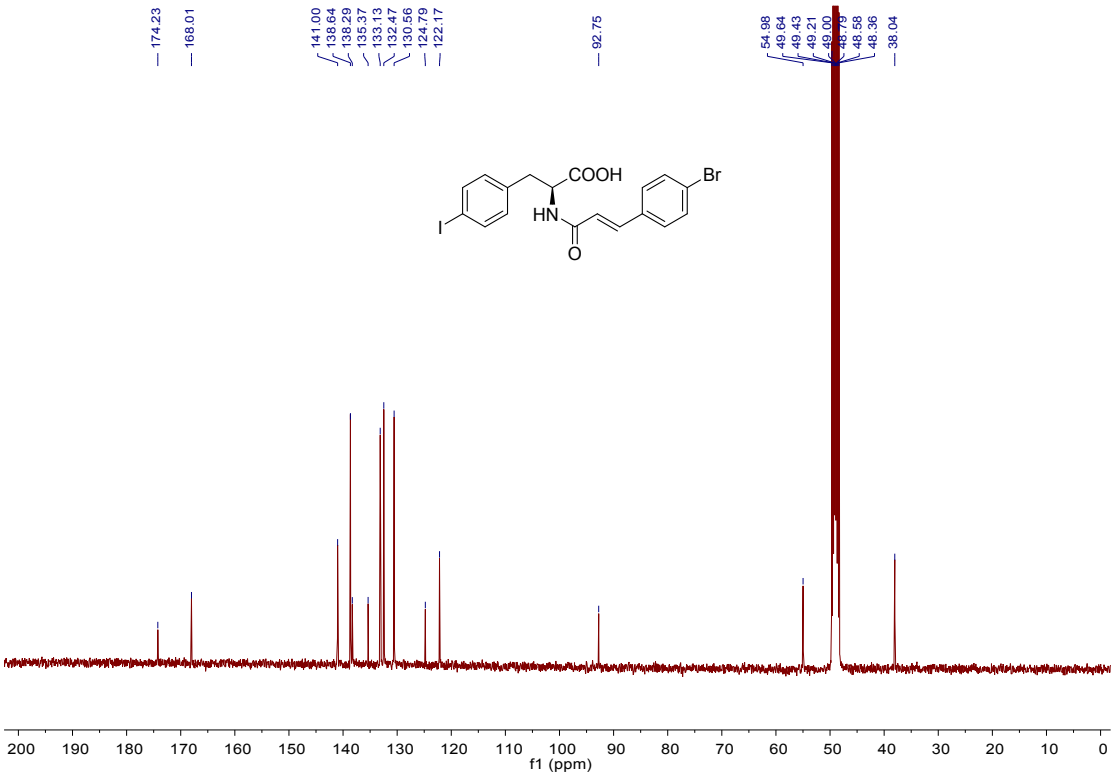


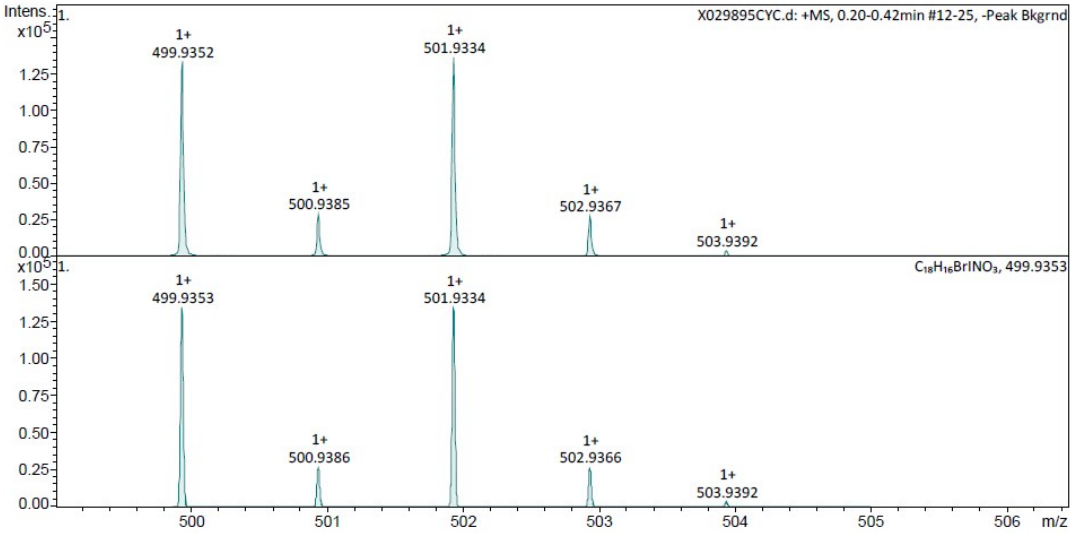



Intens.
x10^5
1.
+MS, 0.15-0.41min #9-24, -Peak Bkgrnd
1+
455.9857
1+
456.9891
1+
457.9835
1+
458.9863
x10^5
1.
C18H16ClINO3, 455.9858
1+
455.9858
1+
456.9891
1+
457.9835
1+
458.9864
456
457
458
459
460
m/z

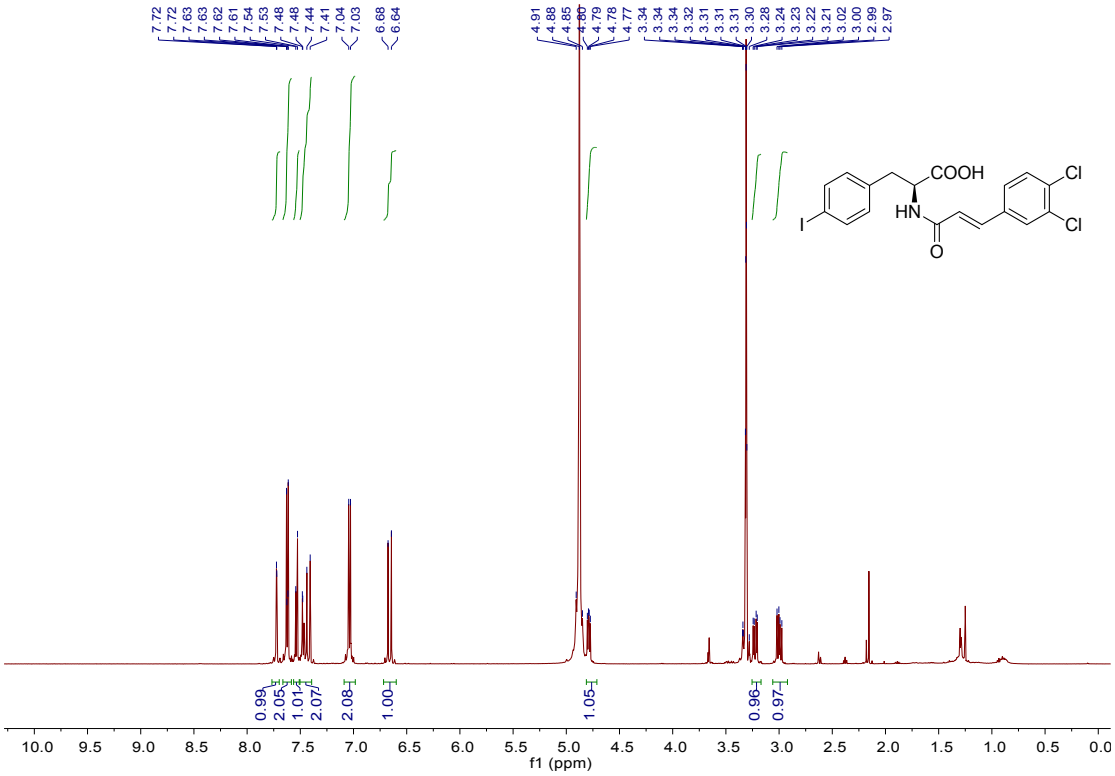
N-(4-Bromocinnamoyl)-4-Iodo-L-phenylalanine (CD_3OD)

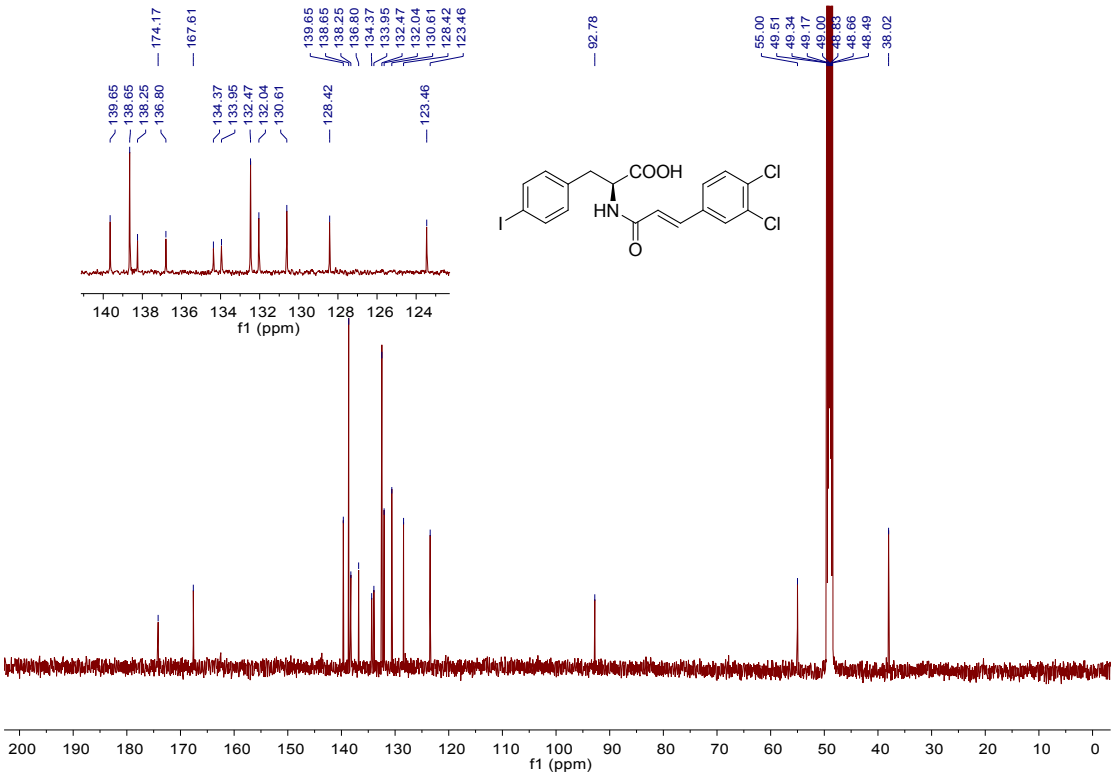


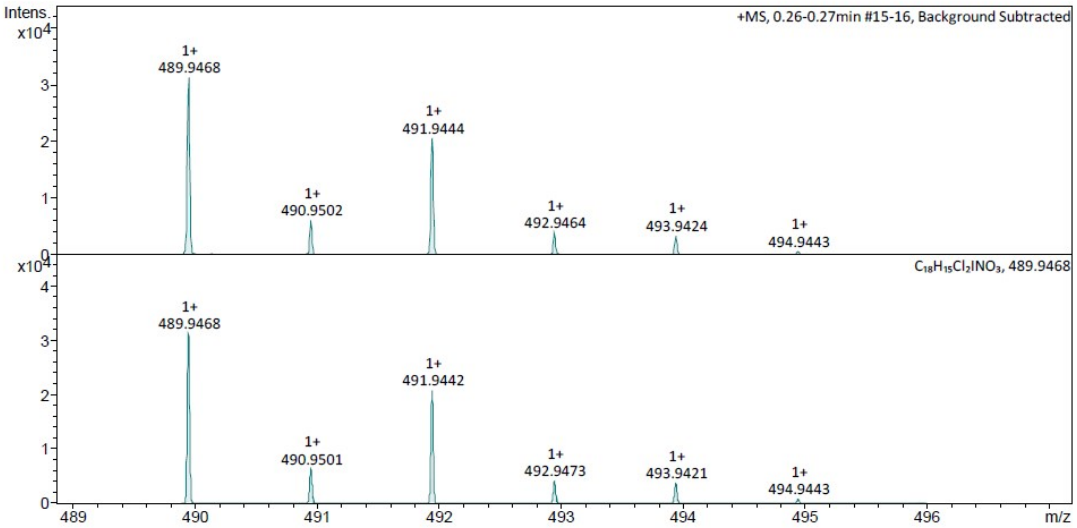



Intens.
x10^5
1.
X029895CYC.d: +MS, 0.20-0.42min #12-25, -Peak Bkgrnd
1+
499.9352
1+
501.9334
1+
500.9385
1+
502.9367
1+
503.9392
1.
C18H16BrINO3, 499.9353
1+
499.9353
1+
501.9334
1+
500.9386
1+
502.9366
1+
503.9392
m/z

N-(3, 4-Dichlorocinnamoyl)-4-Iodo-L-phenylalanine (CD_3OD)






Intens.
x10^4
+MS, 0.26-0.27min #15-16, Background Subtracted
1+
489.9468
1+
490.9502
1+
491.9444
1+
492.9464
1+
493.9424
1+
494.9443
x10^4
C18H15Cl2INO3, 489.9468
1+
489.9468
1+
490.9501
1+
491.9442
1+
492.9473
1+
493.9421
1+
494.9443
489
490
491
492
493
494
495
496
m/z

UGMs preparation

Expression and purification of UGM from *M. tuberculosis*

A vector construct (pET-29b) containing the gene encoding for UGM from *Mycobacterium tuberculosis* was provided by Prof. Laura L. Kiessling. This construct was transformed into BL21(DE3) *E.coli* cells. Transformed cells were grown in Terrific Broth and 50 µg/mL kanamycin at 37°C, culture overnight without induction. Cells were harvested by centrifugation at 6000 rpm for 30 min at 4°C and the pellet was resuspended in the lysis buffer (20 mM sodium phosphate, 25 mM imidazole, 500 mM NaCl, pH 7.4). The disruption of the cells was achieved by lyzozyme, Triton X-100, and sonication. Lysed cells were centrifuged at 16 000 rpm for 50 min at 4°C. The protein was purified by hexahistidine-Ni^{2+}-nitrilotriacetic acid affinity chromatography. After loading of the soluble fraction, the column was washed with a 50 mM phosphate buffer containing 300 mM NaCl and 20 mM imidazole (pH 8). The elution of UGM was made by a linear gradient (0-50%) to 50 mM sodium phosphate buffer (pH 8) containing 300 mM NaCl and 250 mM imidazole. Fractions containing UGM were pooled and dialyzed overnight against 20mM sodium phosphate buffer (pH 7) at 4°C. The purity was estimated by SDS-PAGE and the concentration was measured by absorbance on a spectrophotometer (DTX 880 Multimode Detector) at 450 nm.
UGMs from *K. pneumoniae* is expressed and purified following similar protocols.[1]

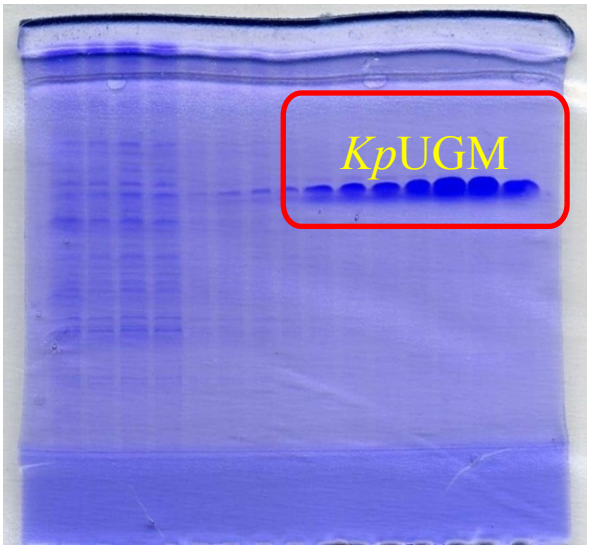


Fig. S8 SDS-page of *Kp*UGM, yield: 101 mg in 1L culture

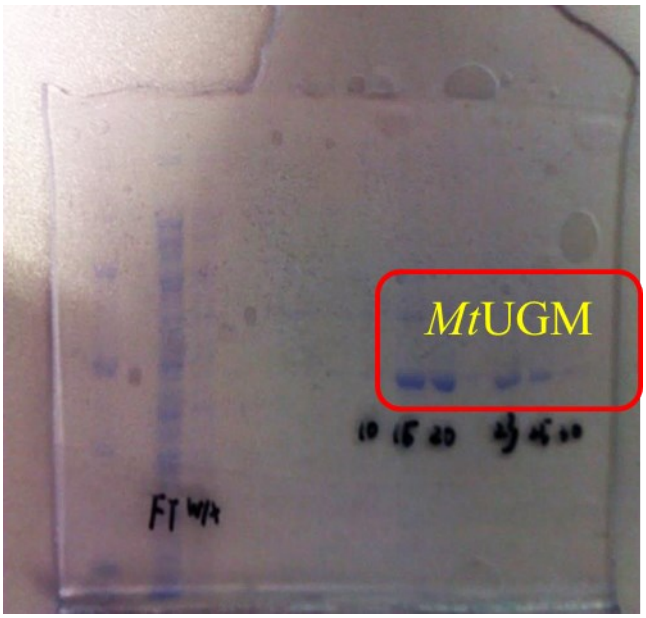


Fig. S9 SDS-page of *Mt*UGM, yield: 11 mg in 1L culture

UGM binding assays

With the enzymes and compounds in hand, we were keen to carry out a biological evaluation. The assay described by Kiessling *et al*. was strictly followed, including the synthesis of the fluorescent probe (UDP-fluorescein).[2-4]

To determine the binding affinity of fluorescent probe towards *Mt*UGM, serial dilutions of dialyzed UGM (final concentration: $1x10^{-5}$ to 12 µM) were incubated with 15 nM of the fluorescent probe in 50 mM sodium phosphate buffer, pH 7.0 at room temperature. Final volumes were 30 µl in 384 well black microtiter plates and the measurements were realized in triplicate. Fluorescence polarization was analyzed using DTX880 Multimode Detector Beckman-Coulter device ($\lambda_{excitation}$ = 485 nm,$\lambda_{emission}$ = 535 nm). Data were fitted to equation (Eq. (4)) with Prism 5 GraphPad Software.

y = FPmin + (FPmax – FPmin)*1/ (1+10^ (log Kd – x)*slope

with y = fluorescence polarization

FPmin = minimal fluorescence polarization signal

FPmax = maximum fluorescence polarization signal

K_d = dissociation constant

x = log [UGM]

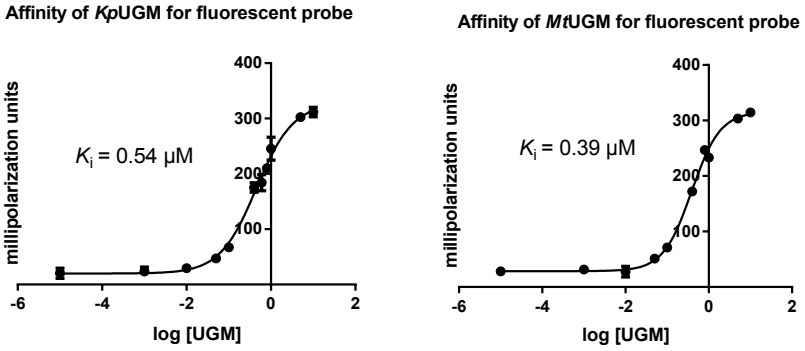


Fig. S10 K_i determination for the fluorescent probe with *Kp*UGM, *Mt*UGM respectively.

Fluorescence polarization assay

Fluorescence polarization binding assays were performed with *Kp*UGM and *Mt*UGM. Serial dilutions of the inhibitor (final concentrations from 0 µM to 1 mM) and 15 nM of the fluorescent probe were mixed in 50 mM phosphate buffer pH 7.0 at room temperature. UGM (final concentration of *Kp*UGM is 500 nM, *Mt*UGM is 580 nM) was added to start the experiment. Final volumes were 30 µl in 384 well black microtiter plates and the measurements were realized in triplicate. Fluorescence polarization was analyzed using DTX880 Multimode Detector Beckman-Coulter device ($\lambda_{excitation}$ = 485 nm,$\lambda_{emission}$ = 535 nm).

Determination of K_d :

Data were fitted to equation (Eq. (5)) with Prism 5 GraphPad Software.

y = FPmin + (FPmax – FPmin)*1/(1+10^(x-log K_d)

log K_d = log (10^ (logK_i * (1+C_f/ K_d f)))

with y = fluorescence polarization,

FPmin = minimal fluorescence polarization signal

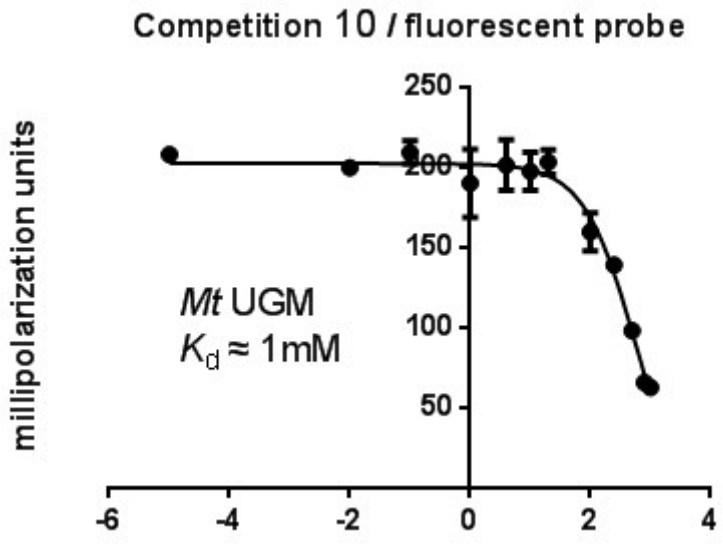
FPmax = maximum fluorescence polarization signal

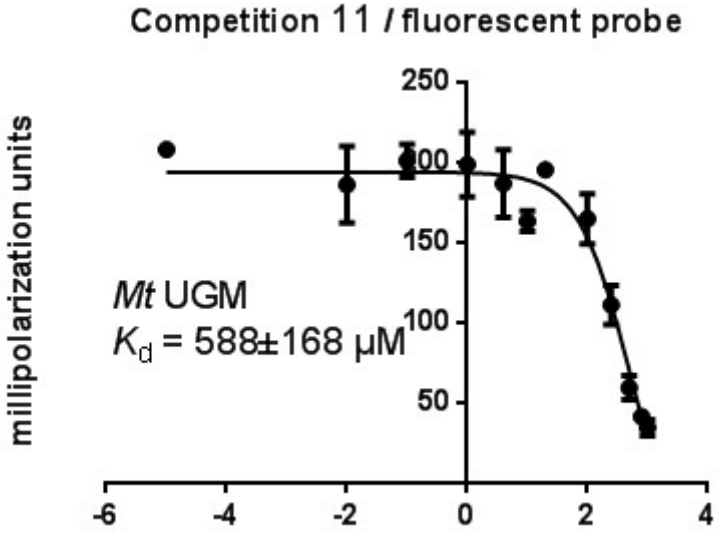
K_d = dissociation constant

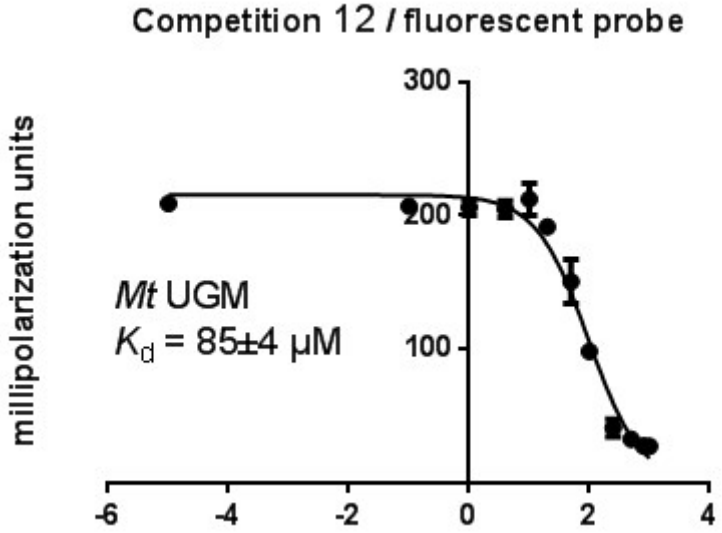
x = log [UGM]

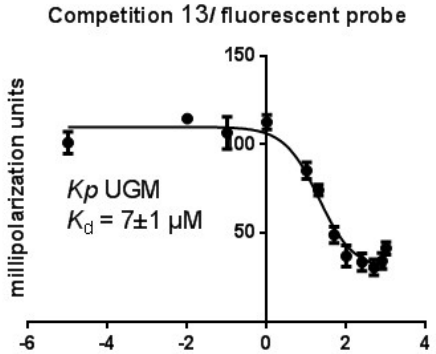
K_i = inhibition constant

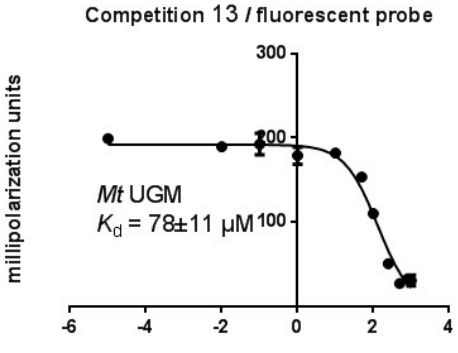
C_f = concentration of the fluorescent probe

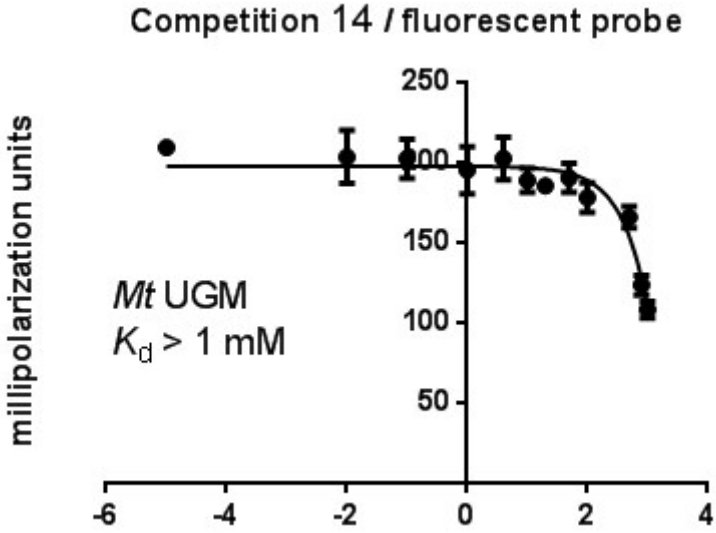


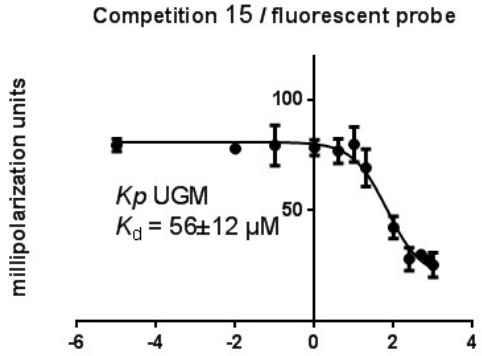

Competition 11 / fluorescent probe
millipolarization units
250
200
150
100
50
Mt UGM
K_d = 588±168 μM
-6
-4
-2
0
2
4

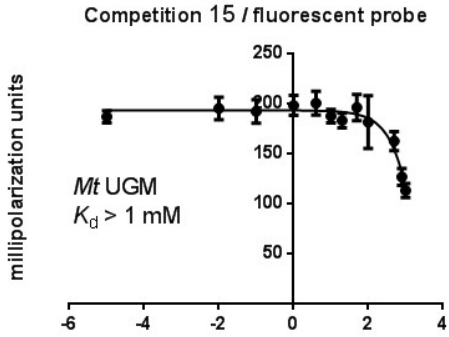

Competition 12 / fluorescent probe
millipolarization units
300
200
100
Mt UGM
K_d = 85±4 μM
-6
-4
-2
0
2
4

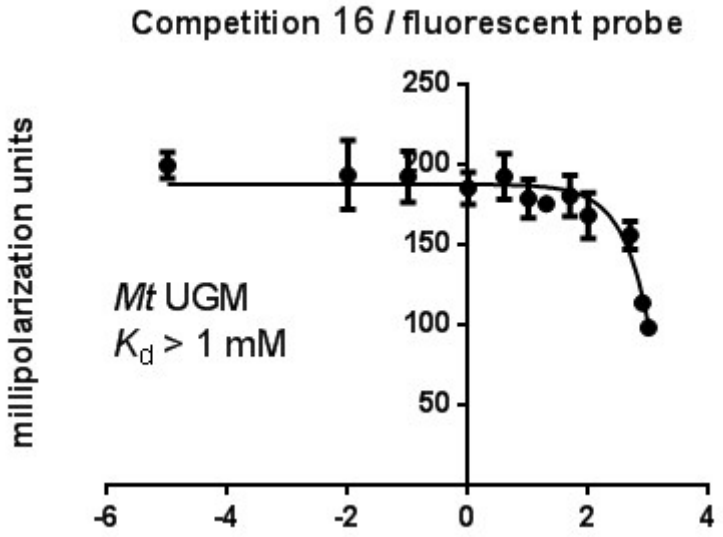

Competition 13/ fluorescent probe
millipolarization units
150
100
50
Kp UGM
K_d = 7±1 μM
-6
-4
-2
0
2
4

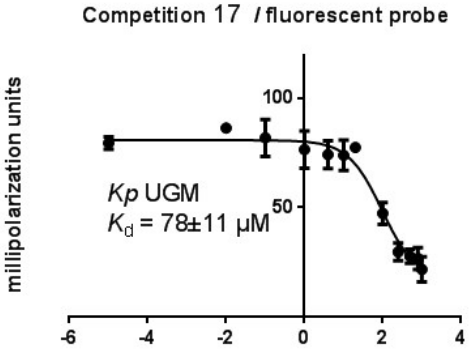

Competition 13 / fluorescent probe
millipolarization units
300
200
100
Mt UGM
K_d = 78±11 μM
-6
-4
-2
0
2
4

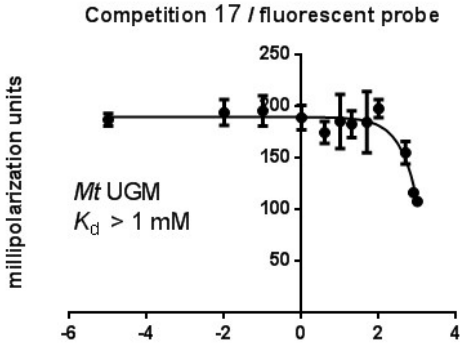

Competition 14 / fluorescent probe
millipolarization units
250
200
150
100
50
-6
-4
-2
0
2
4
Mt UGM
K_d > 1 mM

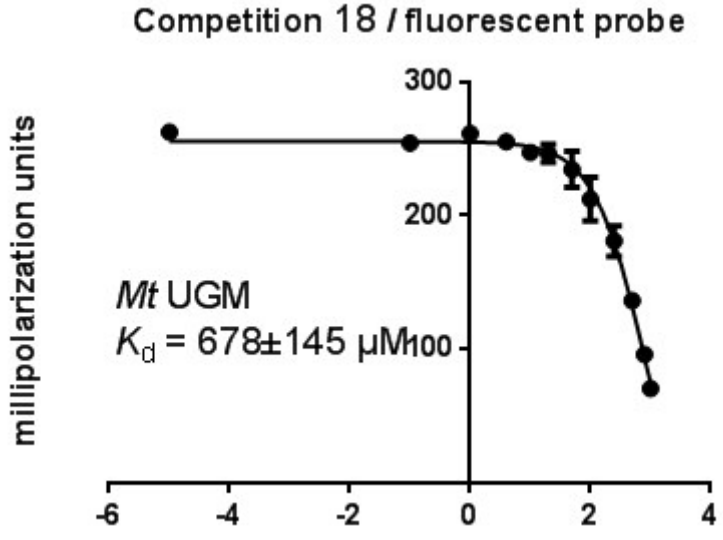

Competition 15 / fluorescent probe
millipolarization units
100
50
-6
-4
-2
0
2
4
Kp UGM
K_d = 56±12 µM

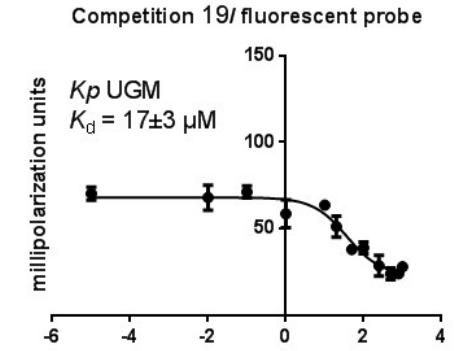

Competition 15 / fluorescent probe
millipolarization units
250
200
150
100
50
-6
-4
-2
0
2
4
Mt UGM
K_d > 1 mM

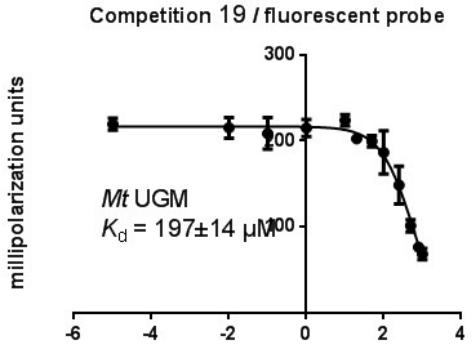

Competition 16 / fluorescent probe
millipolarization units
250
200
150
100
50
-6
-4
-2
0
2
4
Mt UGM
K_d > 1 mM

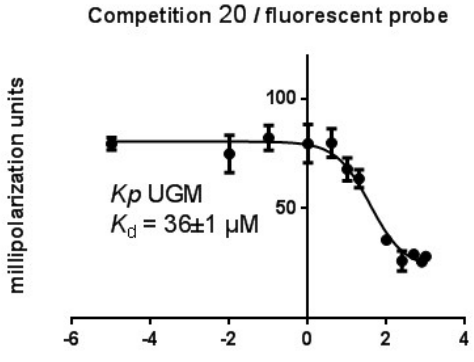

Competition 17 / fluorescent probe
millipolarization units
100
50
-6
-4
-2
0
2
4
Kp UGM
K_d = 78±11 μM

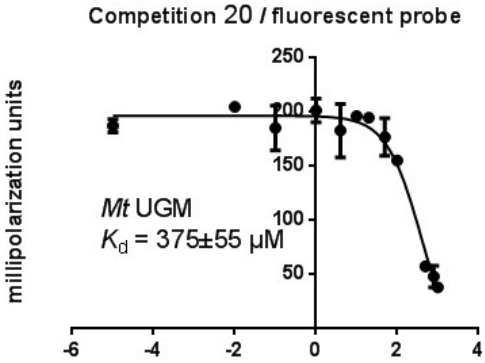

Competition 17 / fluorescent probe
millipolarization units
250
200
150
100
50
-6
-4
-2
0
2
4
Mt UGM
K_d > 1 mM

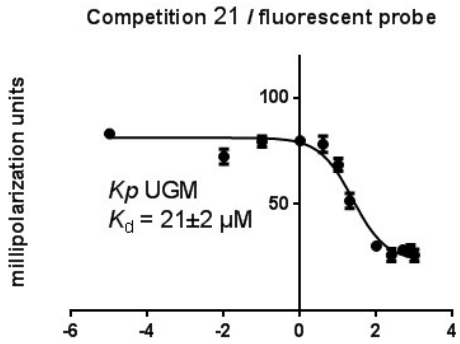

Competition 18 / fluorescent probe
millipolarization units
300
200
100
-6
-4
-2
0
2
4
Mt UGM
K_d = 678±145 μM

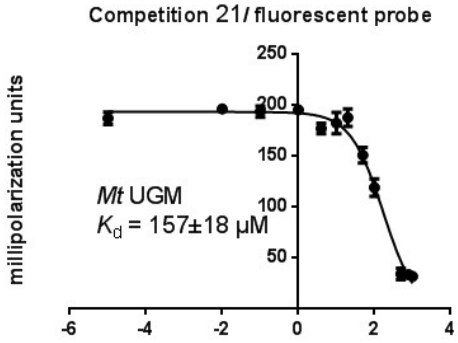

Competition 19/ fluorescent probe
millipolarization units
Kp UGM
Kd = 17±3 μM
150
100
50
-6
-4
-2
0
2
4


Competition 19 / fluorescent probe
millipolarization units
Mt UGM
Kd = 197±14 μM
300
200
100
-6
-4
-2
0
2
4


Competition 20 / fluorescent probe
millipolarization units
Kp UGM
Kd = 36±1 μM
100
50
-6
-4
-2
0
2
4


Competition 20 / fluorescent probe
millipolarization units
Mt UGM
Kd = 375±55 μM
250
200
150
100
50
-6
-4
-2
0
2
4


Competition 21 / fluorescent probe
millipolarization units
Kp UGM
Kd = 21±2 μM
100
50
-6
-4
-2
0
2
4


Competition 21/ fluorescent probe
millipolarization units
Mt UGM
Kd = 157±18 μM
250
200
150
100
50
-6
-4
-2
0
2
4

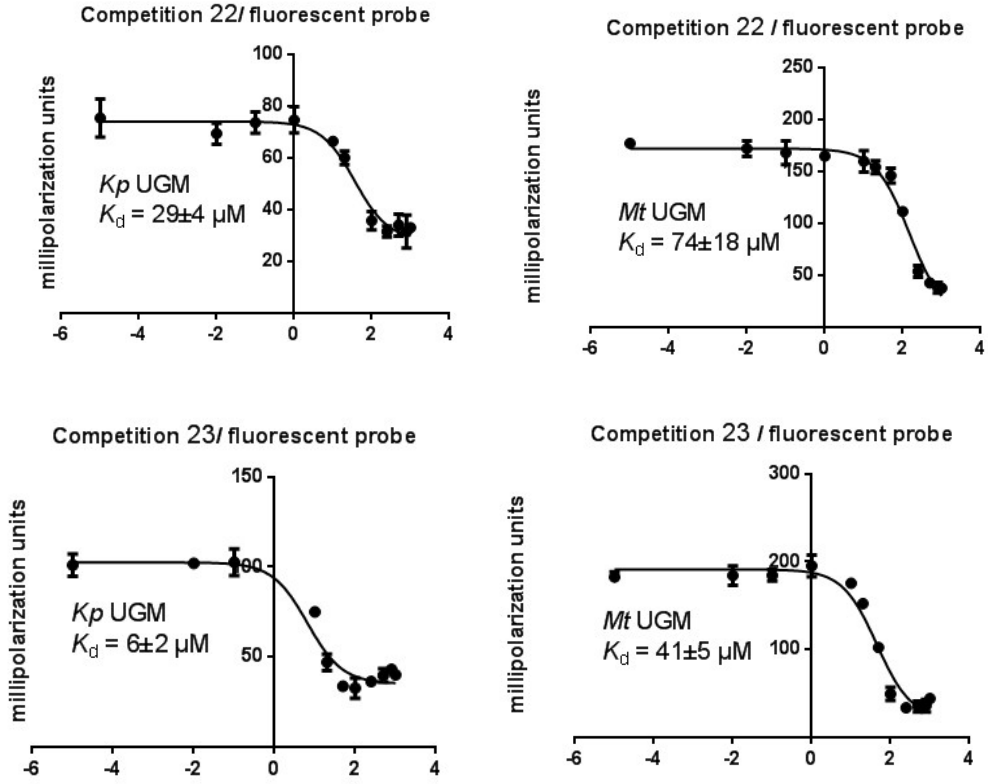


Fig. S11 K_d determination for compounds against *Kp*UGM and *Mt*UGM respectively.

Activity assay / Lineweaver-Burke analysis

The inhibition assay was performed by HPLC. First, added 9 µL blank/ inhibitor (C_f=50 µM), 3 µL *Mt*UGM (C_f=60 nM), reduced with sodium dithionite (C_f=12.5 mM) and incubated for 5 min at room temperature, subsequently added 9 µL UDP-Gla*f* (C_f=25 µM). 6 µL of each reaction was taken and quenched in liquid N_2 at 60, 90, 120, 150, 180 seconds. Injections of the samples were realized by HPLC (Waters 600 E with a C18 Atlantis T3 column, 5 µM 4.6 x 250 mm, elution with 50 mM Triethylamine Acetic Acid, pH 6.8, 0.5% CH_3CN; detection at 262 nm and at a flow rate 1ml/min).

% conversion = (Area UDP-Gal*p* peak)/ [(Area UDP-Gal*p* peak) + (Area UDP-Gal*f* peak)] x 100. By calculating the turnover of inhibited reactions compared to reactions without inhibitor: % inhibition = [(% conversion (without inhibitor) - % conversion (with inhibitor))/ % conversion (without inhibitor)] x 100.

The rate of the conversion of UDP-Gal*f* to UDP-Gal*p* was monitored at several concentrations of substrate (C_f=5, 10, 20, 50, 100 µM) and inhibitor (0, 10, 20, 40µM).

The plots of initial rates versus substrate concentrations were caculated by using Origin8 (Microcal Software, Northhampton, MA) and GraphPad Prism software (GraphPad Software, San Diego, CA). The results were analyzed using a Lineweaver–Burk plot (Figure 1). The fitted lines obtained for the four different concentrations intersect on the Y axis, which is typical competitive inhibition mechanism. The calculated K_i value obtained from the fit was (13.1 ± 3.0) µM.

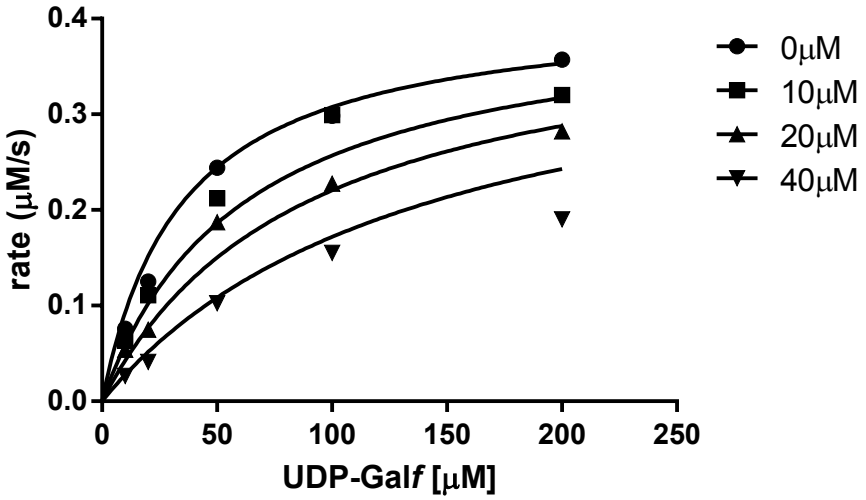


Fig. S12 Plot of the competition assay of molecule 23 against UDP-Gal*f*.

In vitro anti *M. bovis* BCG assay

Step 1 Preparation of inhibitor stock solution in DMSO, concentration is 20000ug/mL= 20mg/mL

Step 2 Preparation of inhibitor mother solution, concentration are 2.5, 5, 25, 50, 250, 500, 1000, 2500, 5000, 10000, 20000 ug/mL

Step 3 Add BCG solution OD?0.6

Step 4 Add inhibitor solution in the 96-well, mix

Step 5 Seal the plate

Step 6 Add 15mL Sauton's medium after 3days

Step 7 Add 15mL Resazurin (0.01% in distilled H2O). The wells were observed after 24 and 48 h for a colour change from blue to pink

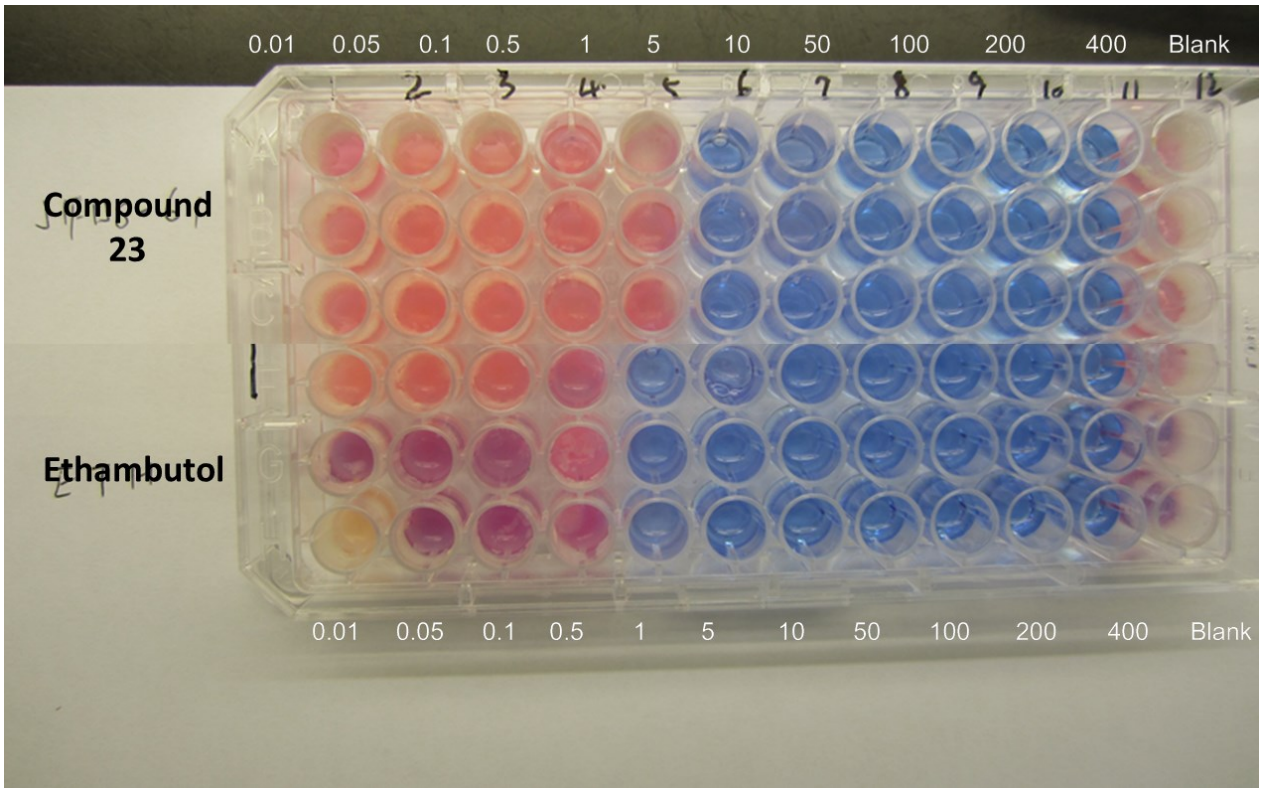


Fig. S13 Compound **23** and ethambutol against *M. bovis* BCG assay

References

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