Supporting Information

Generation of a quenched phosphonate activity-based probe for labelling the active KLK7 protease

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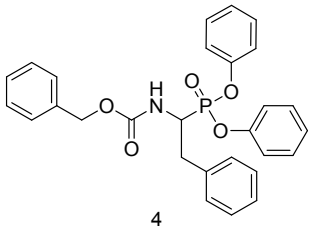
Chemical Synthesis

Benzyl (1-(diphenoxyphosphoryl)-2-phenylethyl)carbamate

Z-Phe^{P}-(OPh)$_2$ (4)



4

Benzyl carbamate **1** (3.02 g, 20 mmol, 1 eq.), 2-phenylacetaldehyde **2** (2.33 ml, 20 mmol, 1 eq.), and triphenyl phosphite **3** (5.25 ml, 20 mmol, 1 eq.) were dissolved in DCM (40 ml) and Cu(OTf)$_2$ (0.722 g, 2 mmol, 0.1 eq.) was added. The mixture was stirred at room temperature (rt) overnight until benzyl carbamate was consumed, as indicated by TLC. Then DCM was evaporated *in vacuo,* and MeOH was added. The resulting solution was kept at 4 °C until diphenyl phosphonate **4** was precipitated as a yellowish solid (5.5 g, yield 56%).

R$_f$: 0.58 (Hexane/EtOAc, 6:4)

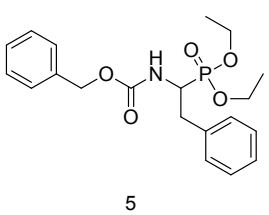
^{1}H-NMR (600 MHz, $CDCl_3$), δ 7.33-7.13 (m, 18H), 7.08 (d, J = 7.9 Hz, 2H), 5.24 (d, J = 10.3 Hz, 1H), 5.03 (s, 2H), 4.90-4.77 (m, 1H), 3.44-3.39 (m, 1H), 3.07-3.01 (m, 1H).

^{31}P-NMR (243 MHz, $CDCl_3$) δ 16.80 (major), 16.37 (minor).

ESI-MS: *m/z* calcd for $[M+Na]^+$ $C_{28}H_{26}NNaO_5P^+$ 510.14, found 510.27; calcd for $[M+K]^+$ $C_{28}H_{26}KNO_5P^+$ 526.12, found 526.22; calcd for $[2M+Na]^+$ $C_{56}H_{52}N_2NaO_{10}P_2^+$ 997.30, found 997.37.

Benzyl (1-(diethoxyphosphoryl)-2-phenylethyl)carbamate

Z-Phe^{P}-(OEt)$_2$ (5)



5

The stirred solution of diphenyl phosphonate **4** (487 mg, 1 mmol, 1 eq), potassium fluoride dihydrate (940 mg, 10 mmol, 10 eq.), and a catalytic amount of 18-crown-6 ether (20 mg, 0.076 mmol, 0.076 eq) in EtOH (5 ml) was heated to reflux for 10 min and then left to cool at rt overnight. The solvent was removed *in vacuo,* and water (20 ml) was added to the residue, followed by extraction with ethyl acetate (3 x 10 ml). The combined organic extracts were washed with 1 N NaOH (3 x 10 ml), water (10 ml), and brine (10 ml), dried over Na_2SO_4, and concentrated *in vacuo* to afford the diethyl phosphonate **5** as a colorless oil (190 mg, yield 49%).

***R**f*: 0.18 (Hexane/EtOAc, 6:4).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.31-7.21 (m, 10H), 5.07 (d, *J* = 9.8 Hz, 1H), 4.99 (s, 2H), 4.42-4.36 (m, 1H), 4.13-3.99 (m, 4H), 3.25-3.21 (m, 1H), 2.88-2.82 (m, 1H), 1.29 (t, *J* = 7.0 Hz, 3H), 1.23 (t, *J* = 7.0 Hz, 3H).

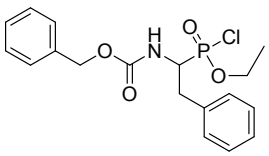
^{13}C NMR (151 MHz, $CDCl_3$) δ 155.7, 136.6, 136.5, 136.3, 129.2, 128.4, 128.1, 127.9, 126.8, 66.9, 62.8, 62.7, 62.5, 62.5, 49.1, 48.1, 36.0, 16.4, 16.3.

^{31}P NMR (243 MHz, $CDCl_3$) δ 23.94 (major), 23.32 (minor).

ESI-MS: *m/z* calcd for $[M+Na]^+$ $C_{20}H_{26}NNaO_5P^+$ 414.14, found 414.15; calcd for $[2M+Na]^+$ $C_{40}H_{52}N_2NaO_{10}P_2^+$ 805.30, found 805.13.

Benzyl (1-(chloro(ethoxy)phosphoryl)-2-phenylethyl)carbamate

Z-Phe^P-(OEt)Cl (6)



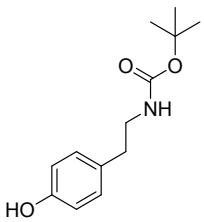
6

To a stirred solution of diethyl phosphonate **5** (194 mg, 0.5 mmol, 1 eq.) in DCM, one drop of DMF was added. The solution cooled at 0 °C, and oxalyl chloride (0.127 ml, 1.5 mmol, 3 eq.) was added dropwise. The mixture was warmed at rt, and the reaction was monitored by TLC. When **5** was consumed (16 h), the solvent was removed *in vacuo*, DCM was added (2 x 5 ml) and evaporated. The residue was used directly to the following reaction without further purification.

***R**f*: 0.26 ($CHCl_3$/MeOH, 9:1).

***Tert*-butyl 4-hydroxyphenethylcarbamate**

Boc-NH-Tya-OH (8)



8

To a stirred solution of tyramine (**7**) (274 mg, 2 mmol, 1 eq.) in dioxane/water (1:1, 10 ml), sodium carbonate (212 mg, 2 mmol, 1 eq.) was added in one portion. The mixture was cooled at 0 °C, and di-*tert*-butyl dicarbonate (Boc_2O) (0.5 ml, 2.2 mmol, 1.1 eq.) was added. One hour after the reaction mixture was left to warm at rt, ethyl acetate (20 ml) was added. The mixture was washed with 5% citric acid (2 x 15 ml), water (15 ml), 5% $NaHCO_3$ (15 ml), and finally with brine (15 ml). The organic layer was dried over Na_2SO_4, and after evaporation, the crude product was purified by column chromatography (silica gel, hexane/ethyl acetate 50% to 20%) to give the desired Boc-protected tyramine **8** as a colorless oil converted to white solid over several days (460 mg, yield 97%).

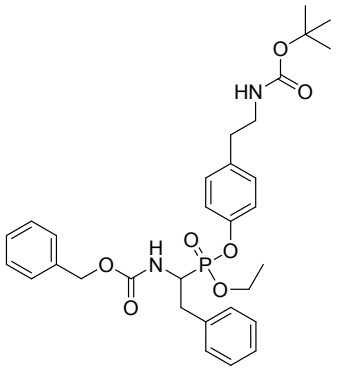
R_f: 0.23 (Hexane/EtOAc, 6:4); 0.44 ($CHCl_3$/MeOH, 9:1).

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.99 (d, J = 8.2 Hz, 2H), 6.79 (d, J = 8.4 Hz, 2H), 4.70 (s, 1H), 3.33-3.32 (m, 2H), 2.70-2.68 (m, 2H), 1.45 (s, 9H).

ESI-MS: *m/z* calcd for $[M+Na]^+$ $C_{13}H_{19}NNaO_3^+$ 260.13, found 260.80.

Benzyl (1-((4-(2-*tert*-butyl carbonyl aminoethyl)phenoxy)(ethoxy)phosphoryl)-2-phenylethyl)carbamate

Z-Phe^P-(OEt)(OTya-Boc) (9)



9

To a stirred solution of chloride **6** (290 mg, 0.76 mmol, 1 eq.) in toluene (2 ml), Boc-NH-Tya-OH (**8**) (450 mg, 1.9 mmol, 2.5 eq.) and triethylamine (0.264 ml, 1.9 mmol, 2.5 eq.) were added. The mixture was stirred at rt overnight. Then diethyl ether (5 ml) was added and extracted with brine (10 ml). The organic layer was washed with 5% $NaHCO_3$ (4 x 5 ml), dried (Na_2SO_4), filtered through celite, and the solvents were evaporated. The crude product was purified in column chromatography (silica gel, hexane/ethyl acetate 80% to 60%) to afford **9** as a white solid (50 mg, 11% after two steps).

R_f: 0.33 (Hexane/EtOAc, 1:1).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.33-7.21 (m, 10H), 7.15-7.08 (m, 4H), 5.16 (t, J = 10.8 Hz, 1H), 5.03 & 4.99 (d, J_{AB} = 24.8 Hz, 2H), 4.20-4.13 (m, 2H), 3.35-3.29 (m, 3H), 2.97-2.95 (m, 1H), 2.77-2.50 (m, 2H), 1.45 (s, 9H), 1.29-1.19 (m, 3H).

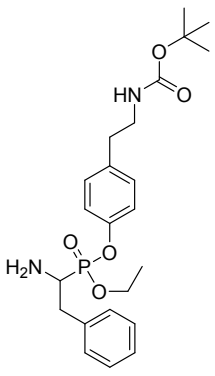
^{13}C NMR (151 MHz, $CDCl_3$) δ 155.8, 155.7, 136.2, 130.1, 129.9, 129.3, 128.5, 128.4, 128.4, 128.1, 128.0, 127.9, 127.8, 126.9, 120.6, 120.4, 67.1, 67.0, 63.7, 63.6, 63.5, 49.6, 48.5, 48.3, 41.7, 36.0, 35.4, 28.4, 16.7, 16.2.

^{31}P NMR (243 MHz, $CDCl_3$) δ 20.84, 20.76.

ESI-MS: *m/z* calcd for $[M+Na]^+$ $C_{31}H_{39}N_2NaO_7P^+$ 605.24, found 605.30; calcd for $[M+K]^+$ $C_{31}H_{39}N_2KO_7P^+$ 621.21, found 621.23.

***Tert*-butyl 4-(((1-amino-2-phenylethyl)(ethoxy)phosphoryl)oxy)phenethylcarba-mate**

H_2N-Phe^P-(OEt)(OTya-Boc) (10)



10

To the stirred mixture of Z-Phe^P-(OEt)(OTya-Boc) (**9**) (50 mg, 86 µmol, 1 eq.) and a catalytic amount of Pd (10% Pd-C, 10 mg, 20%

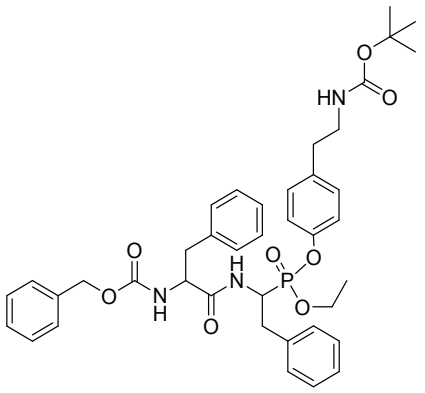
w/w) in MeOH (0.5 ml) triethylsilane (140 µL, 860 µmol, 10 eq.) was added dropwise under nitrogen atmosphere. When the reaction was complete, the mixture was filtered through celite, and solvents evaporated *in vacuo*. The resulting oil was used for the next step without further purification (42 mg crude).

R_f: 0.08 (Hexane/EtOAc, 6:4).

ESI-MS: *m/z* calcd for $[M+H]^+$ $C_{23}H_{34}N_2O_5P^+$ 449.22, found 449.48 .

***Tert*-butyl 4-(((1-(2- benzyloxycarbonylamino-3-phenylpropanamido)-2-phenyl-ethyl)(ethoxy)phosphoryl)oxy)phenethylcarbamate**

Z-Phe-Phe^P-(OEt)(OTya-Boc) (11)



11

To the stirring solution of the amine H_2N-Phe^P-(OEt)(OTya-Boc) (**10**) (38 mg, 0.086 mmol, 1 eq.) in DCM (1 ml), Z-Phe-OH (62 mg, 0.103 mmol, 1.2 eq) was dissolved, followed by the addition of TBTU (78 mg, 0.206 mmol, 2.4 eq), HOBt (27 mg, 0.206 mmol, 2.4 eq.), and DIPEA (44 µL, 0.258 mmol, 3 eq). The mixture was allowed to stir at rt, and DIPEA was added to maintain alkaline pH. When the DPP amine **10** was consumed, the solution was concentrated, ethyl acetate (10 ml) was added, and the mixture was washed with H_2O (4 x 10 ml), 5% $NaHCO_3$ (3 x 10 ml), H_2O (10 ml), 10% citric acid (3 x 10 ml), H_2O (10 ml) and brine (10 ml). The organic phase was dried over Na_2SO_4, and the solvents were removed *in vacuo*. The crude product was purified in column chromatography (silica gel, hexane/ethyl acetate 80% to 60%) to give **11** as a white solid (30 mg, 47% after two steps).

R_f: 0.54 (Hexane/EtOAc, 6:4).

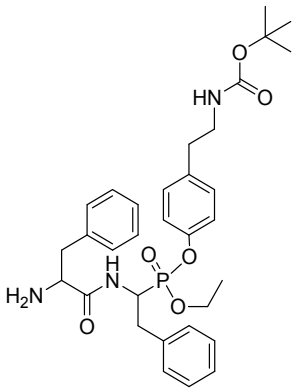
^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.35-7.06 (m, 19H), 6.98 (d, J = 3.4 Hz, 1H), 6.51-6.47 (m, 1H), 5.06-5.04 (m, 2H), 4.95-7.90 (m, 1H), 4.58-4.52 (m, 1H), 4.35-4.25 (m, 1H), (4.14-4.04 (m, 2H), 3.34-3.27 (m, 3H), 3.02-2.70 (m, 5H), 1.45 & 1.44 (s, 9H, isomers).

^{31}P-NMR (243 MHz, $CDCl_3$) δ 20.36, 20.27, 20.22.

ESI-MS: *m/z* calcd for $[M+Na]^+$ $C_{40}H_{48}N_3NaO_8P^+$ 752.31, found 752.42; calcd for $[M+K]^+$ $C_{40}H_{48}KN_3O_8P^+$ 768.28, found 768.38.

***Tert*-butyl 4-(((1-(2-amino-3-phenylpropanamido)-2-phenylethyl)(ethoxy)phosphoryl)oxy)phenethylcarbamate**

H_2N-Phe-Phe^P-(OEt)(OTya-Boc) (12)



12

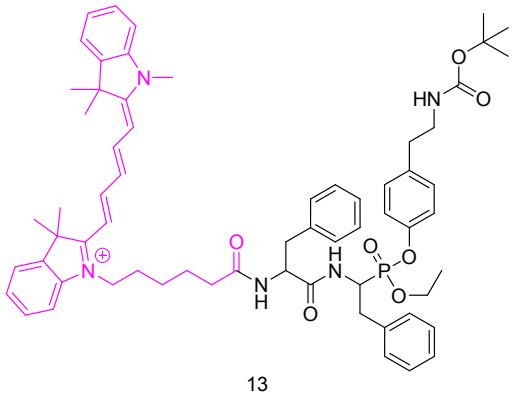
To the mixture of Z-Phe-Phe^P-(OEt)(OTya-Boc) (**11**) (20 mg, 27 µmol, 1 eq.) and 10% Pd-C (4 mg, 20% w/w) in MeOH (0.5 ml), was added triethylsilane (44 µL, 270 µmol, 10 eq.) dropwise under nitrogen atmosphere. When the reaction was complete, the mixture was filtered through celite, and solvents evaporated *in vacuo*. The crude oil was used without further purification (16 mg crude).

***R**$_f$*: 0.08 (Hexane/EtOAc, 6:4).

ESI-MS: *m/z* calcd for $[M+H]^+$ $C_{32}H_{43}N_3O_6P^+$ 596.29, found 596.37.

1-(6-((1-((1-((4-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenoxy)(ethoxy)phosphoryl)-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1*E*,3*E*,5*E*)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium

Cy5-Phe-Phe^P-(OEt)(OTya-Boc) (13)



13

To a solution of **12** (8 mg, 13 µmol, 1 eq.) in 150 µL DMSO, a solution of Cy5-NHS (10 mg, 15.6 µmol, 1.2 eq.) in 150 µL DMSO was added, followed by the addition of DIPEA (11 µL, 8.5 µmol, 5 eq.). The reaction was monitored with HPLC till **12** was consumed. After 16 h, purification by HPLC (semi-preparatory reverse phase C_{18} column, CH_3CN/H_2O + 0.1% TFA, 25% for 3 min; 25% to 100% over 15 min, 1 ml/min) followed by lyophilization, afforded pure the TFA salt of product **13** as a blue powder (3.7 mg, yield 25% after two steps).

^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.5 Hz, 1H), 7.44 (d, *J* = 8.3 Hz, 1H), 7.56 (t, *J* = 8.4 Hz, 1H), 7.49 (t, *J* = 8.4 Hz, 1H), 7.44-7.07 (m, 23H), 4.26-4.21 (m, 1H) 4.15-4.04 (m, 4H), 3.67-3.62 (m, 1H), 3.57-3.54 (m, 1H), 3.46 (s, 3H), 2.35 (t, *J* = 7.4 Hz, 6H), 2.26 (t, *J* = 6.0 Hz, 6H), 2.01 (d, *J* = 5.5 Hz, 2H), 1.71-1.59 (m, 6H), 1.40 (s, 9H), 1.14-1.19 (m, 3H). 6H peaks overlap with residual solvent peaks.

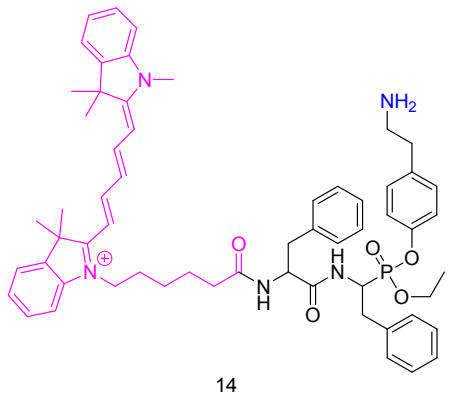
t_R: peak 1: 30.34 min (70.66% B), peak 2: 30.59 (71.15% B) (diasteromers); CH_3CN/H_2O + 0.1% TFA, 20% for 5 min; 20% to 100% over 40 min, 1 ml/min.

ESI-MS: *m/z* calcd for $[M]^+$ $C_{64}H_{79}N_5O_7P^+$ 1060.57, found 1060.86; calcd for $[M+Na]^{2+}/2$ $C_{64}H_{79}N_5NaO_7P^{2+}$ 541.78, found 541.93. Both HPLC peaks gave the same ESI-MS.

1-(6-((1-((1-((4-(2-aminoethyl)phenoxy)(ethoxy)phosphoryl)-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1*E*,3*E*,5*E*)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium

Cy5-Phe-Phe^P-(OEt)(OTya-NH_2) (14)

To a stirred solution of **13** (2 mg, 1.7 µmol, 1 eq.) in DCM (0.1 ml), was added dropwise 0.1 ml TFA. After 2 h, the mixture was co-evaporated with toluene (3 x 0.3 ml) *in vacuo* on a rotavap to dryness. The crude amorphous TFA salt was used in the next reaction without further purification (2 mg crude).



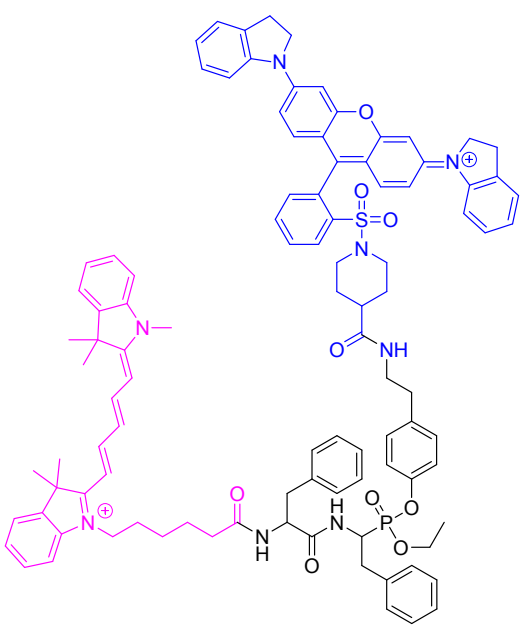
14

t_R: peak 1: 23.08min (56.10% B), peak 2: 23.45 min (56.57% B) (diastereomers); CH_3CN/H_2O + 0.1% TFA, 20% for 5 min; 20% to 100% over 40 min, 1 ml/min.

ESI-MS: *m/z* calcd for $[M]^+$ $C_{59}H_{71}N_5O_5P^+$ 960.52, found 960.72; calcd for $[(M+H)/2]^{2+}$ 480.76, found 480.91.

1-(6-((1-((1-(ethoxy(4-(2-(1-((2-((*E*)-3-(indolin-1-ium-1-ylidene)-6-(indolin-1-yl)-3*H*-xanthen-9-yl)phenyl)sulfonyl)piperidine-4-carboxamido)ethyl)phenoxy)phosphoryl)-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1*E*,3*E*,5*E*)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium

Cy5-Phe-Phe^P-(OEt)(OTya-QSY21) (15)



15

To a solution of **14** (2 mg, 1.7 µmol, 1 eq.) in DMSO (50 µL), a solution of QSY21-NHS (1.6 mg, 1.91 µmol, 1.1 eq.) in 50 µL DMSO was added, followed by the addition of DIPEA (1.5 µL, 8.5 µmol, 5 eq.). The reaction was monitored by RP-HPLC till **14** was consumed. After 16 h, purification by RP-HPLC (semi-preparative reverse phase C_{18} column, CH_3CN/H_2O + 0.1% TFA, 25% for 3 min; 25% to 100% over 15 min, 1 ml/min) followed by lyophilization, afforded pure the TFA salt of product **15** as a dark blue powder (1.9 mg, yield 65% for two reactions).

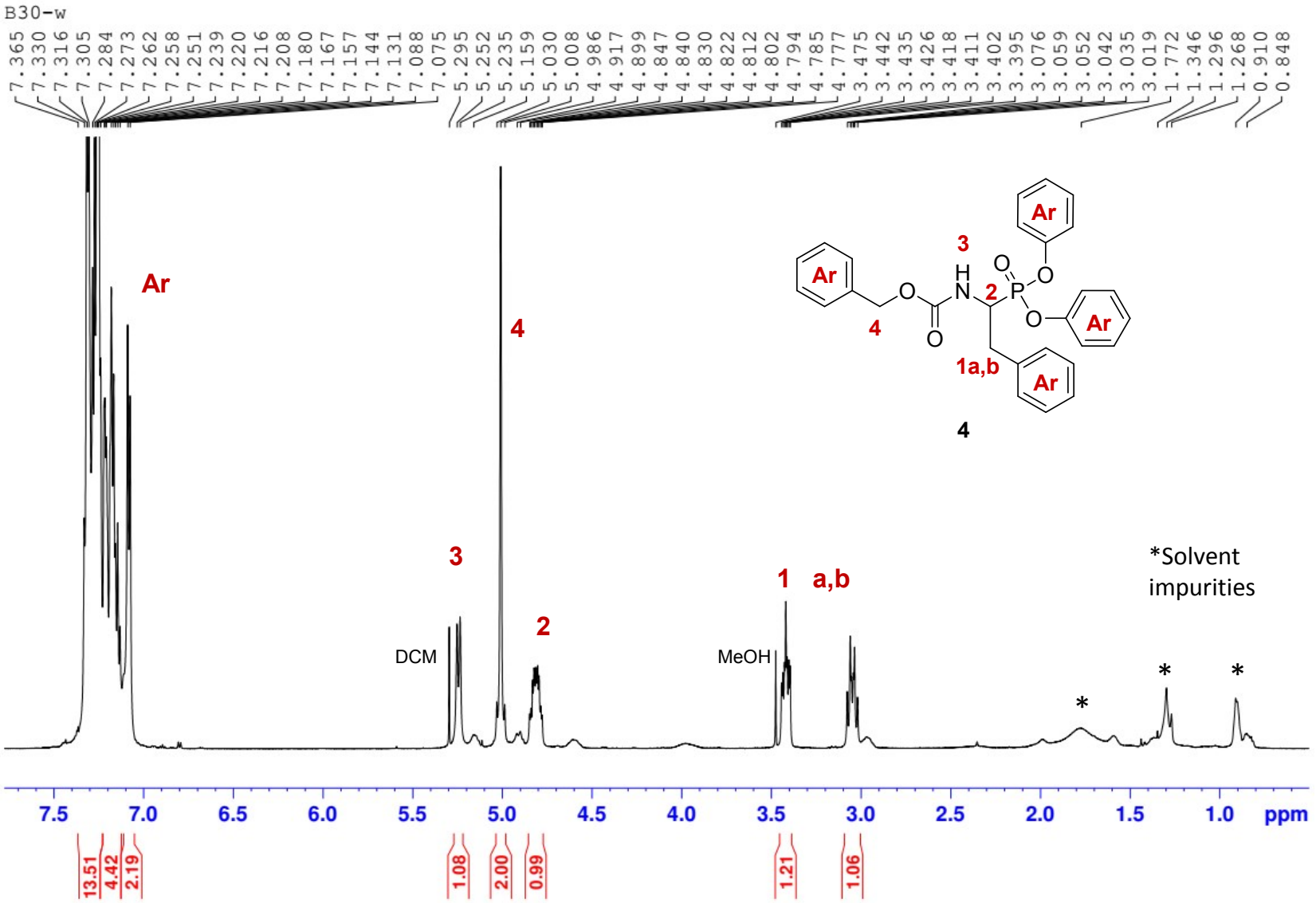
^{1}H-NMR (600 MHz, $CDCl_3$) δ 8.24-8.20 (m, 2H), 7.96-7.92 (m, 2H), 7.88 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H), 7.67-7.46 (m, 10H), 7.39-7.01 (m, 26H), 6.76 (d, J = 8.4 Hz, 1H), 4.34-4.07 (m, 9H), 3.75-3.73 (m, 2H), 3.57 (s, 3H) 3.19-3.18 (m, 2H), 2.58-2.53 (m, 6H), 2.36-2.06 (m, 6H), 2.05-1.98 (m, 2H), 1.85-1.69 (m, 8H), 1.65-1.45 (m, 6H), 1.24-1.23 (m, 3H). 10H peaks overlap with residual solvent peaks.

t_R: peak 1: 29.30 min (69.48% B), peak 2: 30.18 min (69.95% B) (diastereomers); CH_3CN/H_2O + 0.1% TFA, 20% for 5 min; 20% to 100% over 40 min, 1 ml/min).

ESI-MS: *m/z* calcd for $[M]^{2+}/2$ $C_{100}H_{105}N_8O_9PS^{2+}$ 812.87, found 812.96; calcd for $[M+H]^{3+}/3$ 542.25, found 542.33.

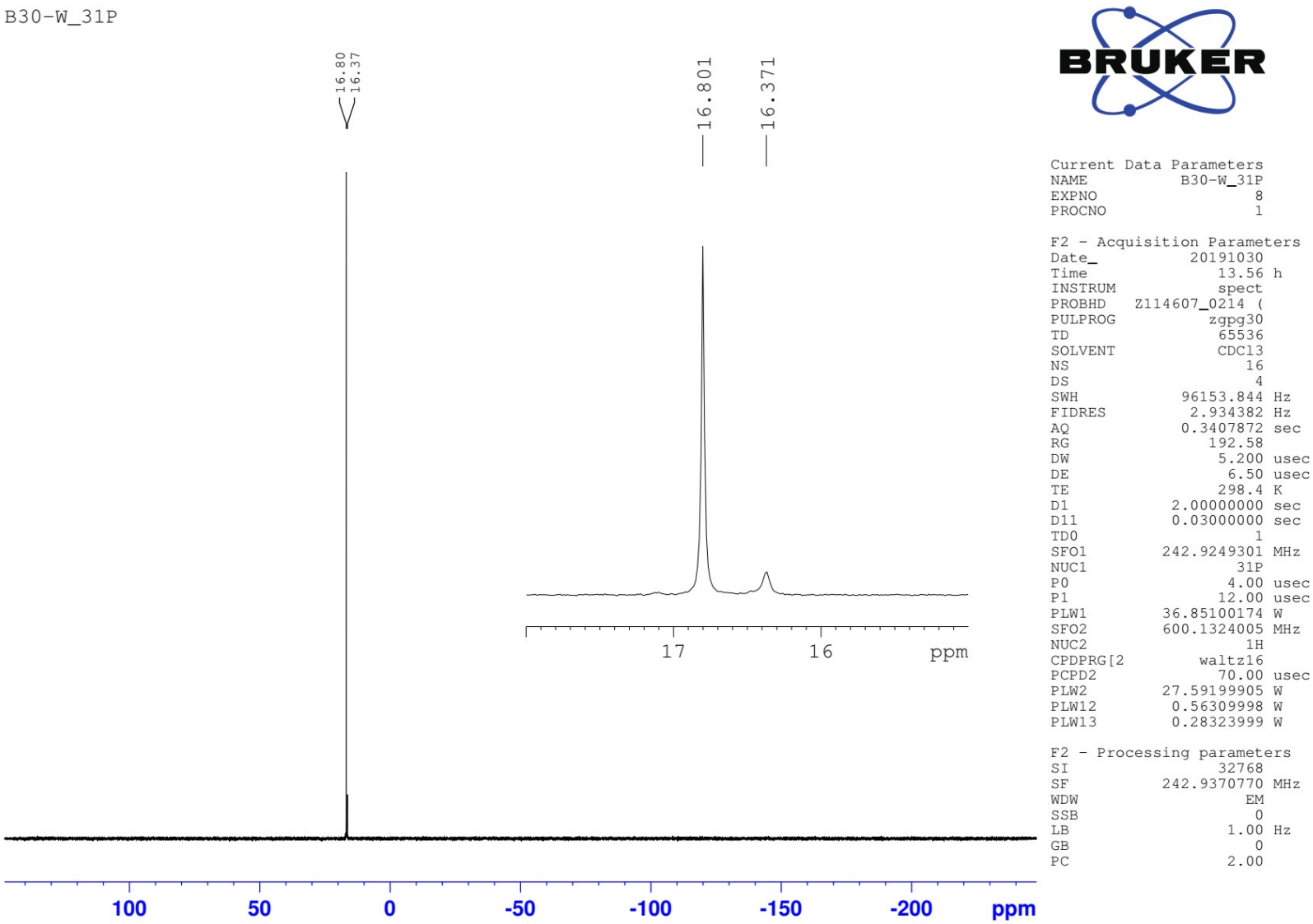
NMR, MS and HPLC raw data

^{1}H NMR (600 MHz, $CDCl_3$) of compound Z-Phe^{P}-$(OPh)_2$ (**4**).



4

^{31}P NMR (243 MHz, $CDCl_3$) of compound Z-Phe^{P}-$(OPh)_2$ (**4**).

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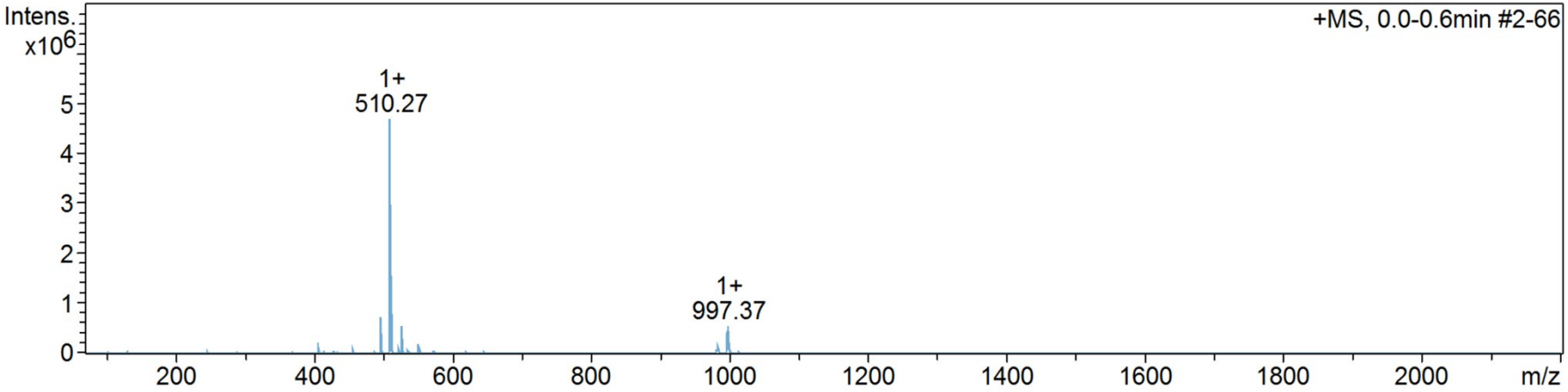
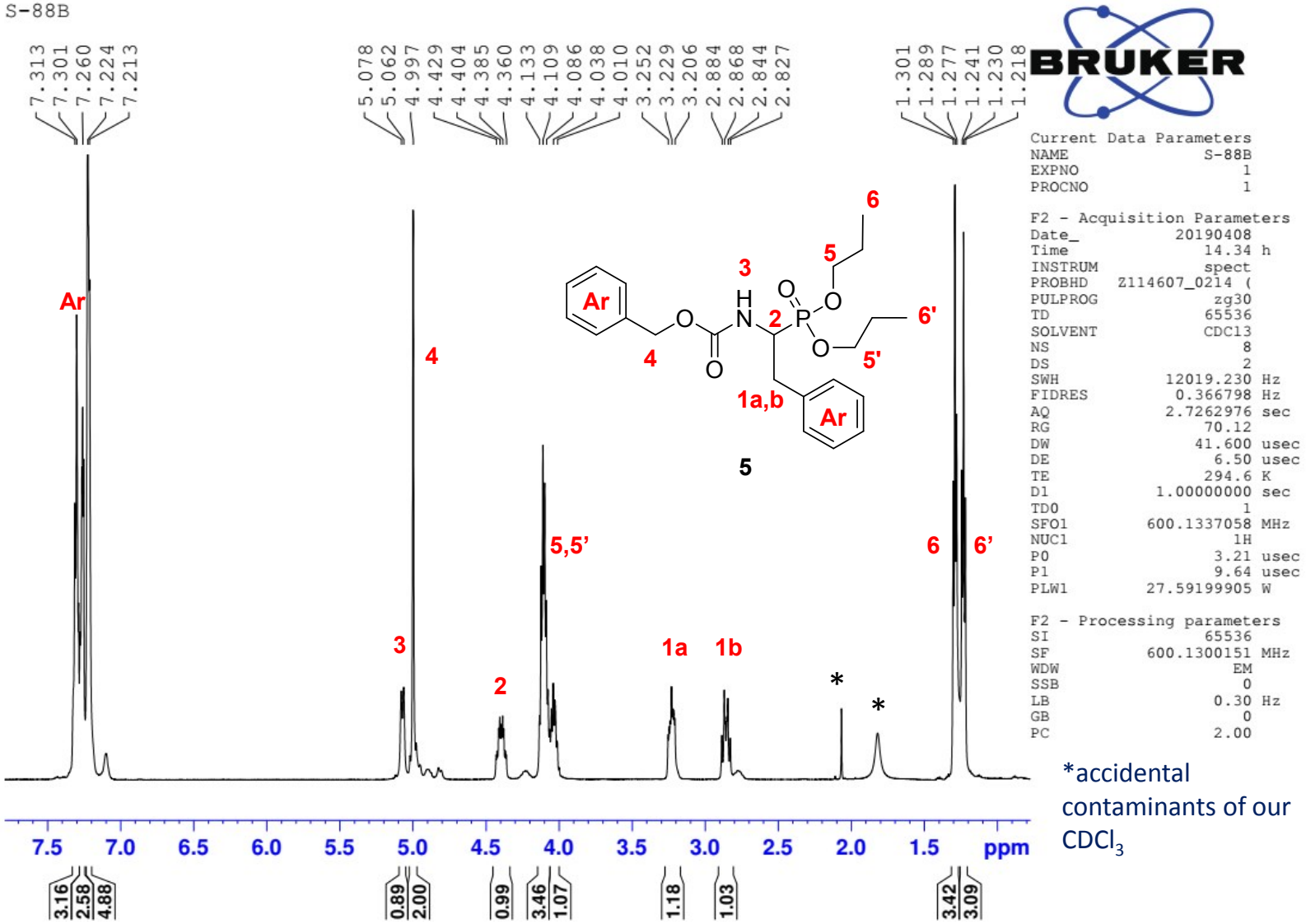
ESI-MS of compound Z-Phe^{P}-$(OPh)_2$ (**4**).



m/z calcd for $[M+Na]^+$ $C_{28}H_{26}NNaO_5P^+$ 510.14, found 510.27; calcd for $[M+K]^+$ $C_{28}H_{26}KNO_5P^+$ 526.12, found 526.22; calcd for $[2M+Na]^+$ $C_{56}H_{52}N_2NaO_{10}P_2^+$ 997.30, found 997.37.

^{1}H NMR (600 MHz, $CDCl_3$) of compound Z-Phe^{P}-$(OEt)_2$ (**5**).



5

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Current Data Parameters
NAME                  S-88B
EXPNO                     1
PROCNO                    1

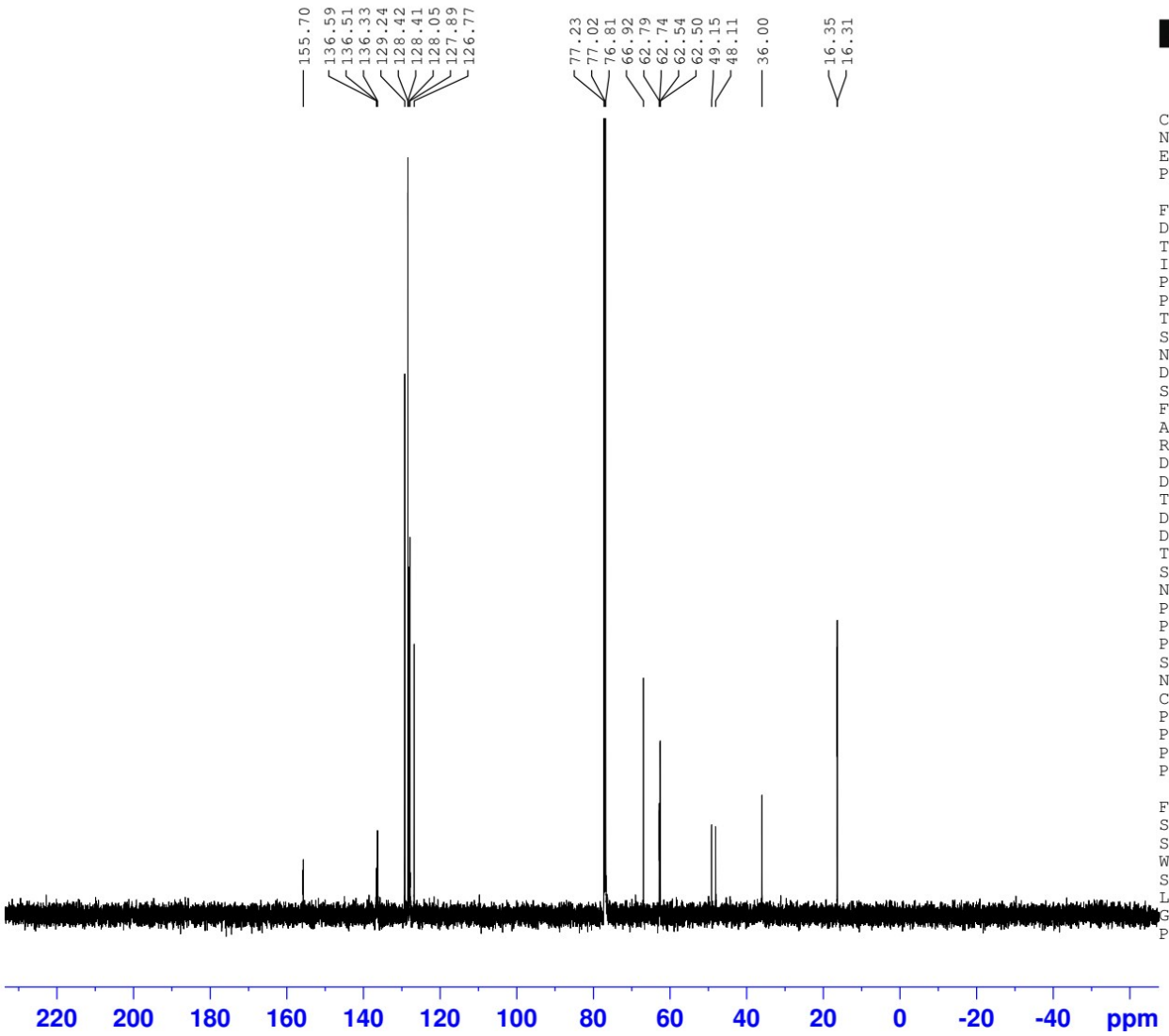
F2 - Acquisition Parameters
Date_              20190408
Time                 14.34 h
INSTRUM              spect
PROBHD    Z114607_0214 (
PULPROG               zg30
TD                   65536
SOLVENT              CDC13
NS                       8
DS                       2
SWH              12019.230 Hz
FIDRES           0.366798 Hz
AQ               2.7262976 sec
RG                   70.12
DW                 41.600 usec
DE                   6.50 usec
TE                  294.6 K
D1              1.00000000 sec
TD0                      1
SFO1           600.1337058 MHz
NUC1                    1H
P0                   3.21 usec
P1                   9.64 usec
PLW1           27.59199905 W

F2 - Processing parameters
SI                   65536
SF             600.1300151 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    2.00
```

*accidental contaminants of our $CDCl_3$

^{13}C NMR (151 MHz, $CDCl_3$) of compound Z-Phe^P-$(OEt)_2$ (**5**).

S88-B_13C

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Current Data Parameters
NAME          S88-B_13C
EXPNO                 1
PROCNO                1

F2 - Acquisition Parameters
Date_          20190409
Time             13.16 h
INSTRUM           spect
PROBHD   Z114607_0214 (
PULPROG         zgpg30
TD               65536
SOLVENT          CDCl3
NS                 214
DS                   0
SWH          45454.547 Hz
FIDRES        1.387163 Hz
AQ           0.7208960 sec
RG              192.58
DW              11.000 usec
DE               6.50 usec
TE              296.9 K
D1          2.00000000 sec
D11         0.03000000 sec
TD0                  1
SFO1        150.9153389 MHz
NUC1               13C
P0               4.00 usec
P1              12.00 usec
PLW1        75.48300171 W
SFO2        600.1324000 MHz
NUC2               1H
CPDPRG[2        waltz16
PCPD2            70.00 usec
PLW2        27.59199905 W
PLW12        0.55530000 W
PLW13        0.27831000 W

F2 - Processing parameters
SI               32768
SF          150.9028107 MHz
WDW                EM
SSB                 0
LB               1.00 Hz
GB                  0
PC               1.40
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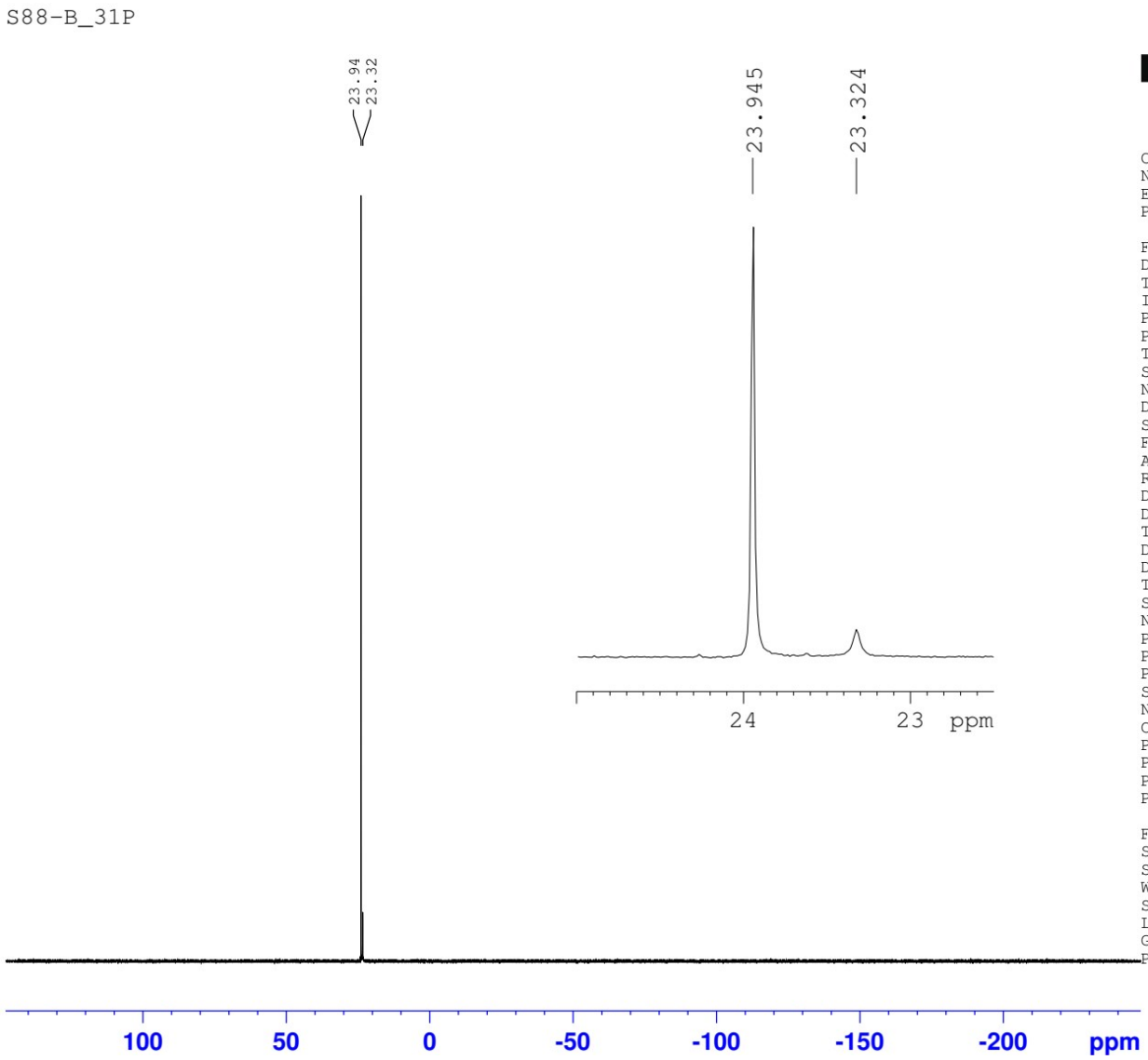
^{31}P NMR (243 MHz, $CDCl_3$) of compound Z-Phe^{P}-$(OEt)_2$ (**5**).

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Current Data Parameters
NAME          S88-B_31P
EXPNO                 1
PROCNO                1

F2 - Acquisition Parameters
Date_          20190409
Time              13.04 h
INSTRUM           spect
PROBHD   Z114607_0214 (
PULPROG         zgpg30
TD                65536
SOLVENT           CDCl3
NS                   11
DS                    4
SWH           96153.844 Hz
FIDRES         2.934382 Hz
AQ            0.3407872 sec
RG               192.58
DW                5.200 usec
DE                6.50 usec
TE                296.6 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1
SFO1        242.9249301 MHz
NUC1               31P
P0                4.00 usec
P1               12.00 usec
PLW1        36.85100174 W
SFO2        600.1324005 MHz
NUC2               1H
CPDPRG[2        waltz16
PCPD2            70.00 usec
PLW2        27.59199905 W
PLW12        0.55530000 W
PLW13        0.27831000 W

F2 - Processing parameters
SI                32768
SF          242.9370770 MHz
WDW                EM
SSB                   0
LB                 1.00 Hz
GB                    0
PC                 2.00
```
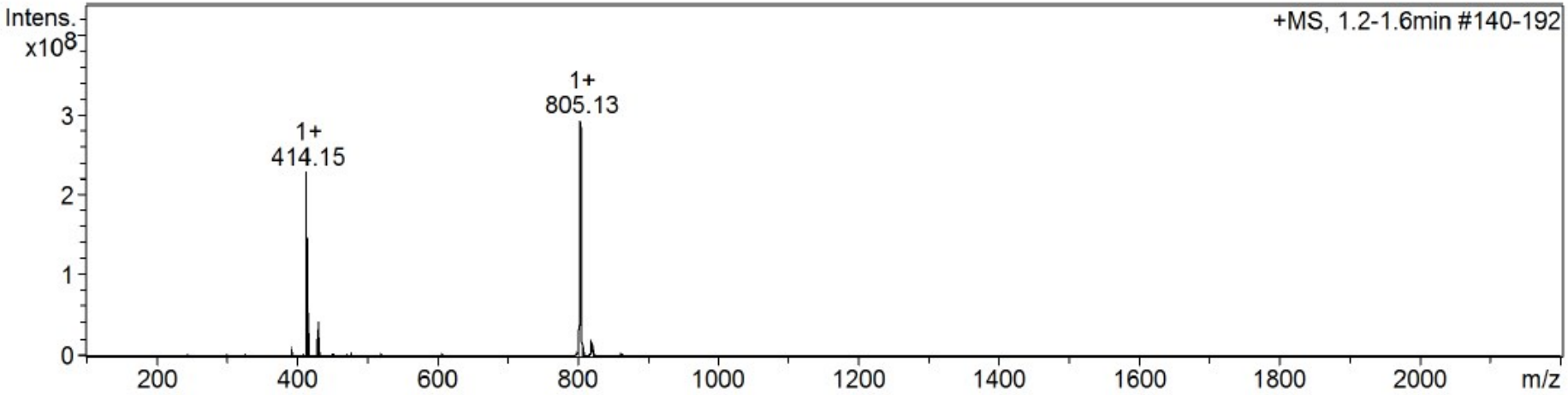
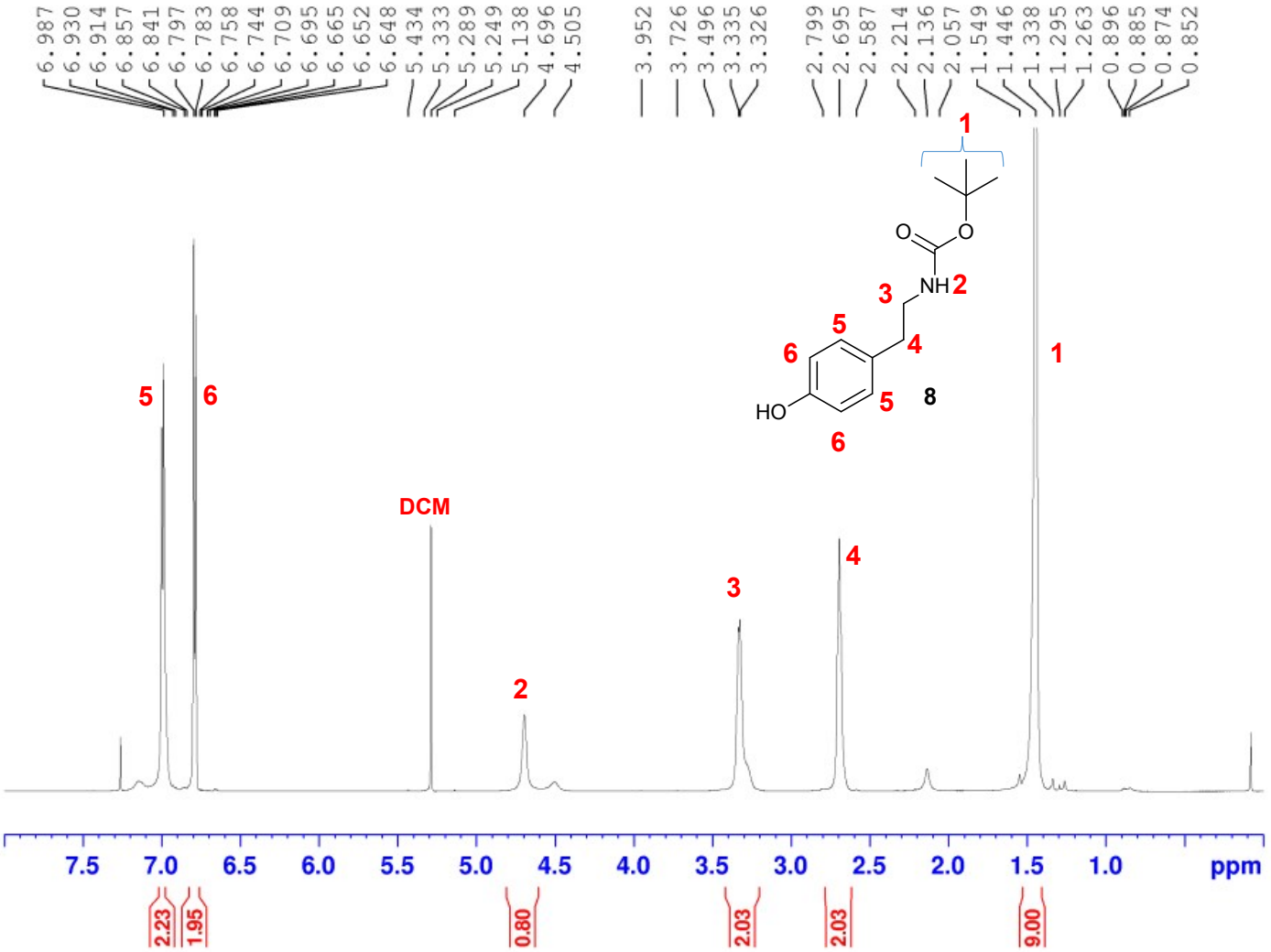

ESI-MS of compound Z-Phe^{P}-$(OEt)_2$ (**5**).



m/z calcd for $[M+Na]^+$ $C_{20}H_{26}NNaO_5P^+$ 414.14, found 414.15; calcd for $[2M+Na]^+$ $C_{40}H_{52}N_2NaO_{10}P_2^+$ 805.30, found 805.13.

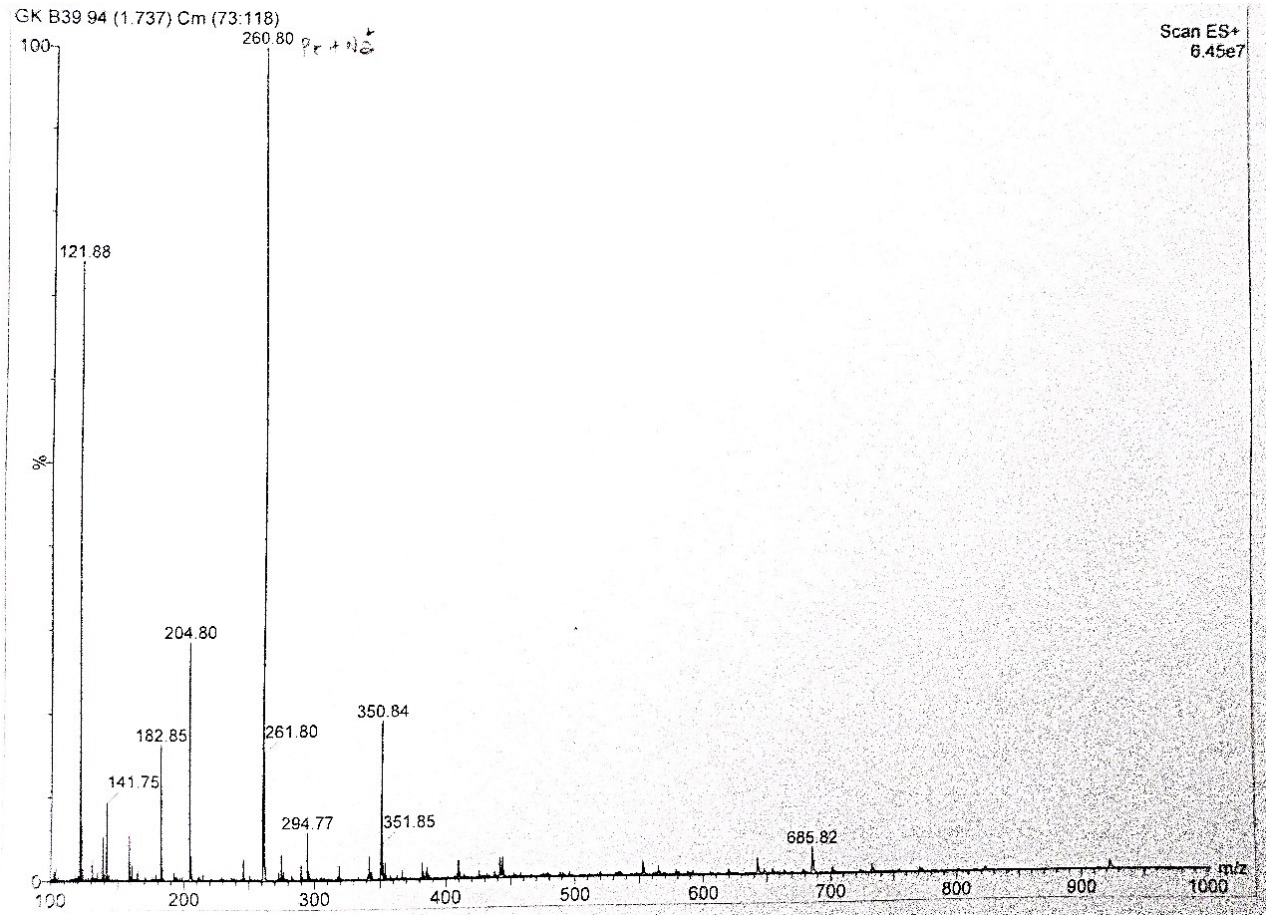
^{1}H NMR (600 MHz, 298 K, $CDCl_3$) of compound Boc-NH-Tya-OH (**8**).

B44c5



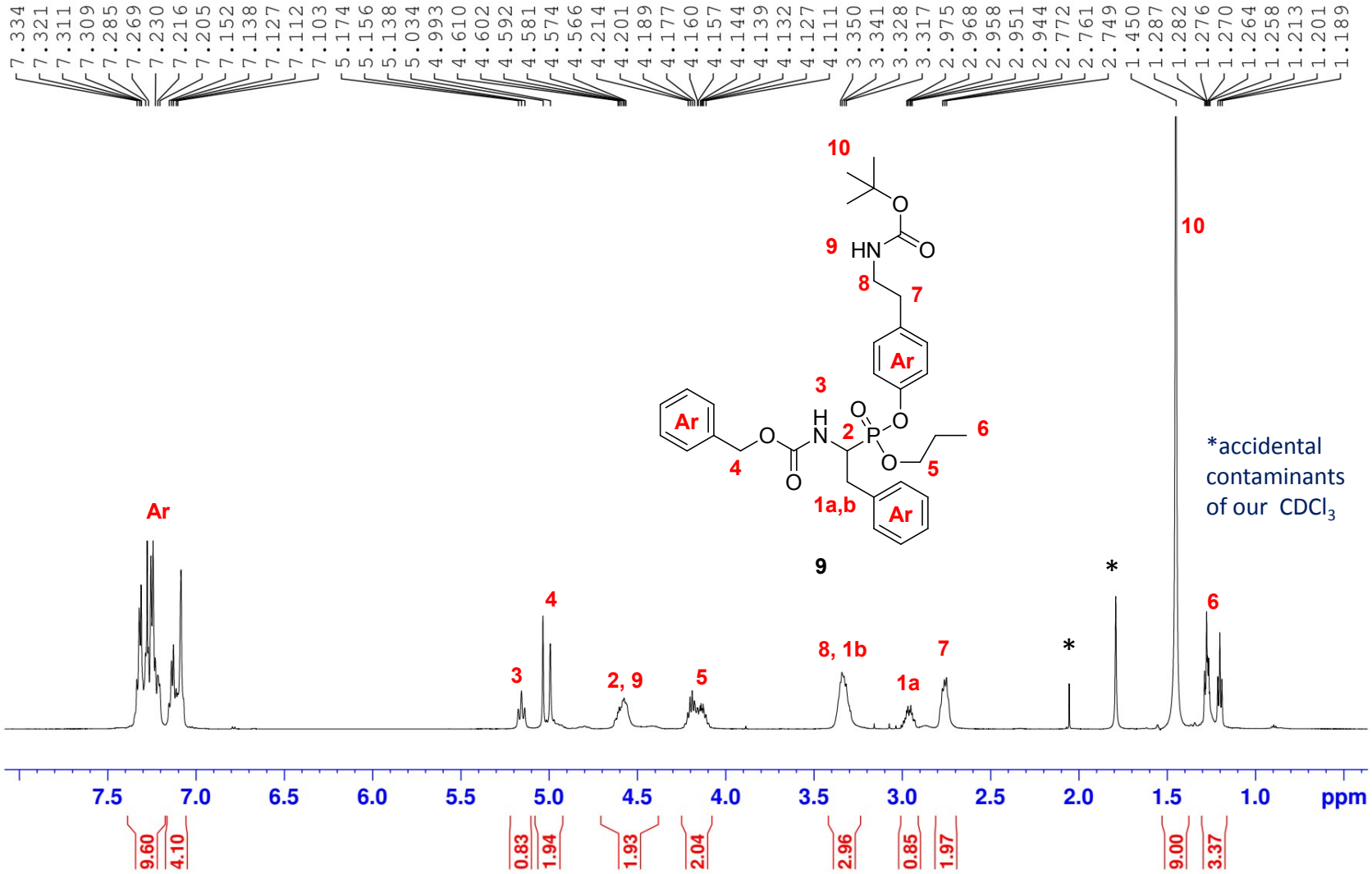

```
Current Data Parameters
NAME                  B44c5
EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
Date_              20191113
Time                 16.55 h
INSTRUM              spect
PROBHD    Z114607_0214 (
PULPROG              zg30
TD                  65536
SOLVENT             CDCl3
NS                     11
DS                      2
SWH             12019.230 Hz
FIDRES              0.366798 Hz
AQ                2.7262976 sec
RG                   22.48
DW                  41.600 usec
DE                   6.50 usec
TE                  298.1 K
D1             1.00000000 sec
TD0                     1
SFO1          600.1337058 MHz
NUC1                   1H
P0                   3.55 usec
P1                  10.66 usec
PLW1          27.59199905 W

F2 - Processing parameters
SI                  65536
SF            600.1300134 MHz
WDW                    EM
SSB                     0
LB                   0.30 Hz
GB                      0
PC                   4.00
```

ESI-MS of compound Boc-NH-Tya-OH (**8**).

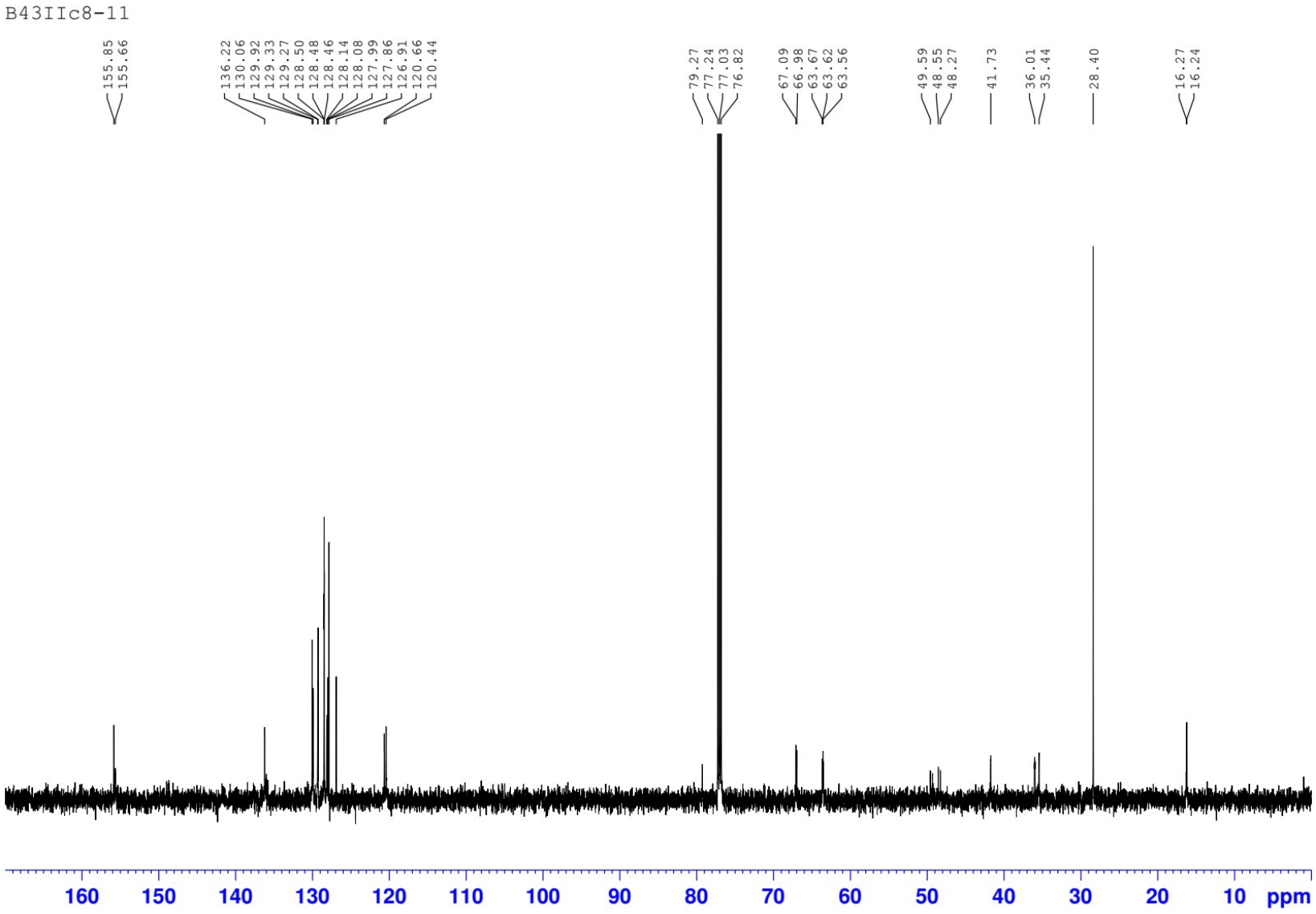


m/z calcd for $[M+Na]^+$ $C_{13}H_{19}NNaO_3^+$ 260.13, found 260.80.

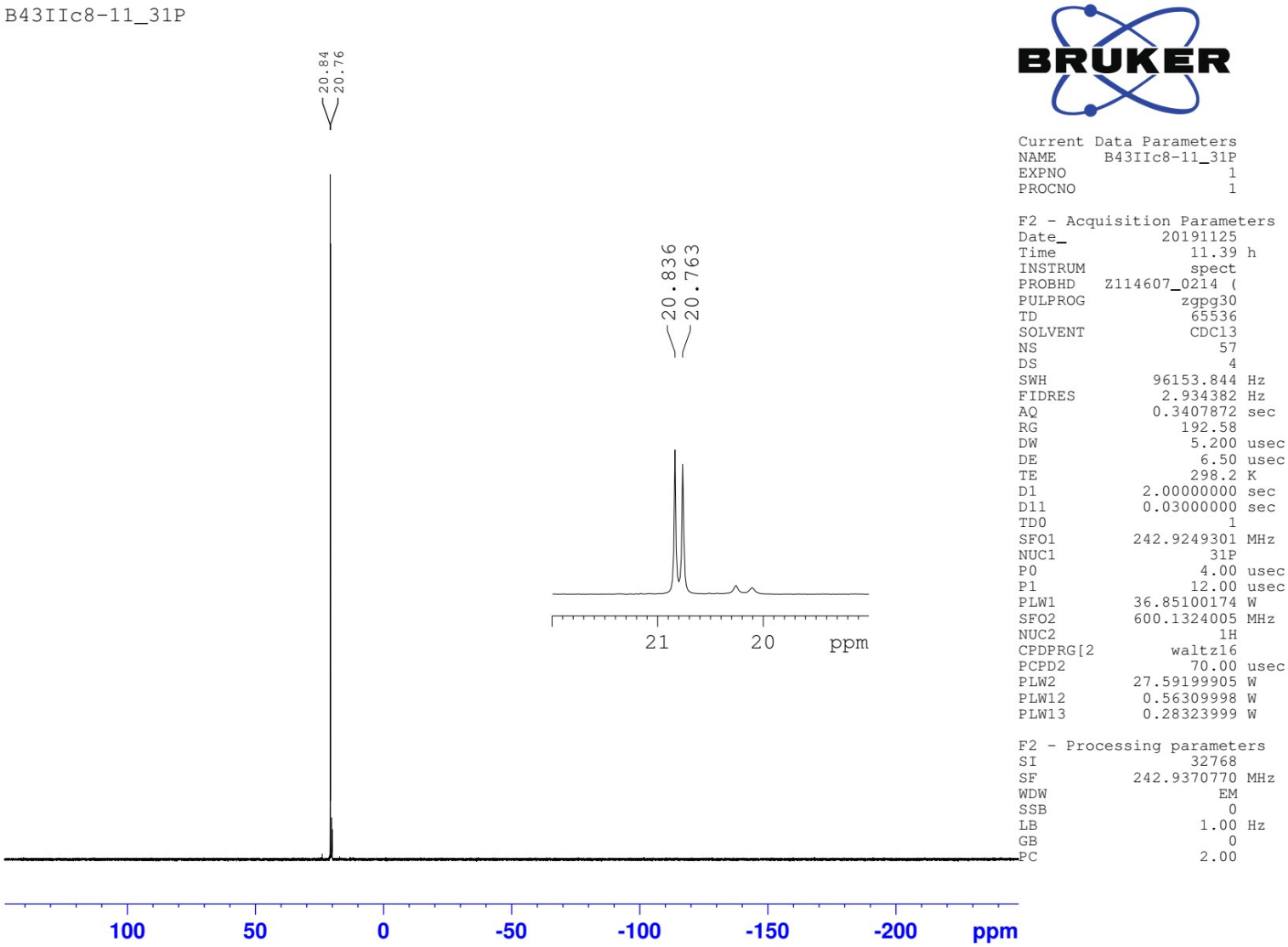
^{1}H NMR (600 MHz, $CDCl_3$) of compound Z-Phe^{P}-(OEt)(OTya-Boc) (**9**).



^{13}C NMR (151 MHz, $CDCl_3$) of compound Z-Phe^{P}-(OEt)(OTya-Boc) (**9**).



^{31}P NMR (243 MHz, $CDCl_3$) of compound Z-Phe^{P}-(OEt)(OTya-Boc) (**9**).

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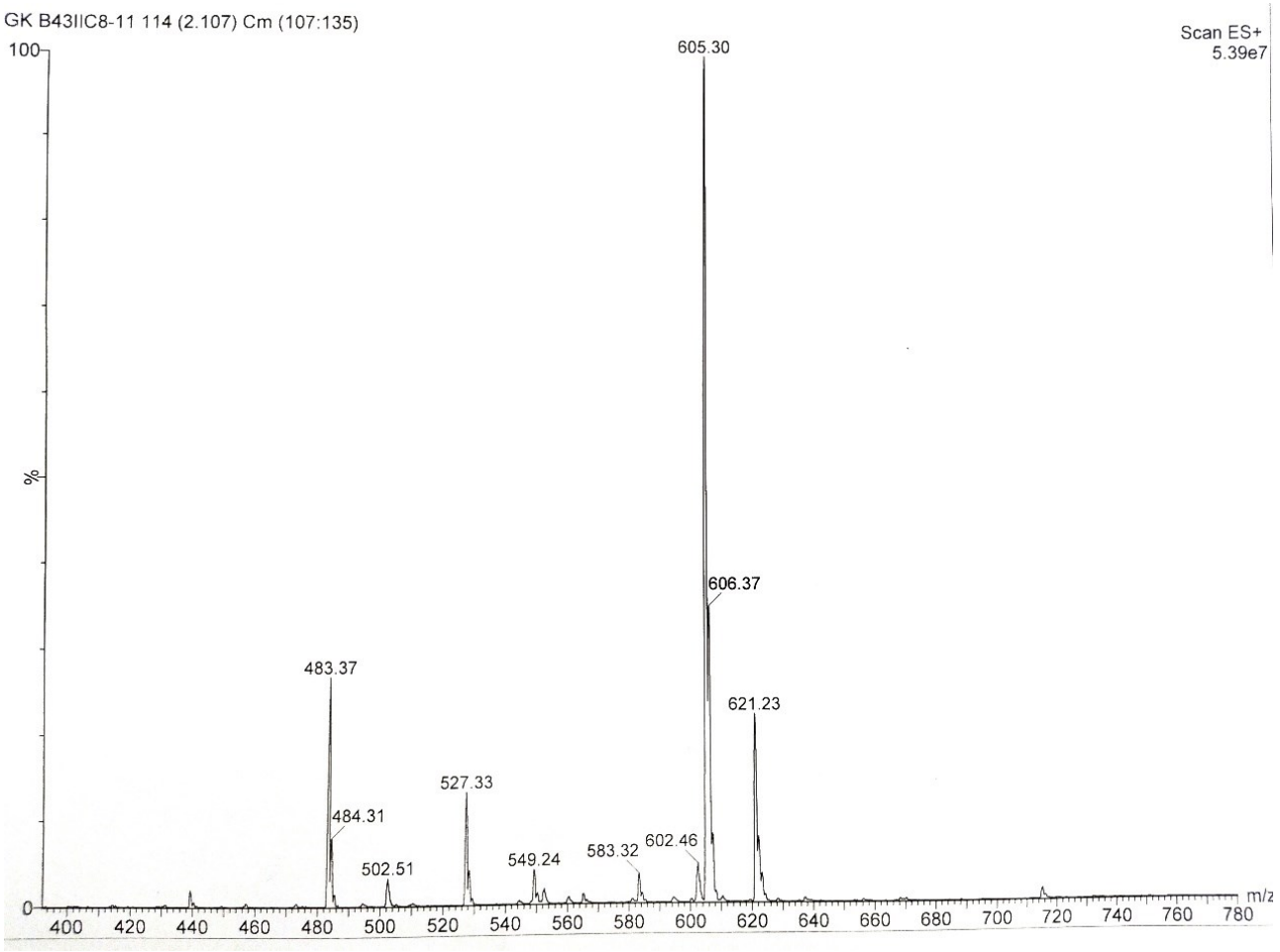
Current Data Parameters
NAME      B43IIc8-11_31P
EXPNO                 1
PROCNO                1

F2 - Acquisition Parameters
Date_          20191125
Time              11.39 h
INSTRUM            spect
PROBHD   Z114607_0214 (
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                   57
DS                    4
SWH           96153.844 Hz
FIDRES         2.934382 Hz
AQ            0.3407872 sec
RG               192.58
DW                5.200 usec
DE                6.50 usec
TE                298.2 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1
SFO1        242.9249301 MHz
NUC1               31P
P0                4.00 usec
P1               12.00 usec
PLW1        36.85100174 W
SFO2        600.1324005 MHz
NUC2               1H
CPDPRG[2        waltz16
PCPD2             70.00 usec
PLW2        27.59199905 W
PLW12        0.56309998 W
PLW13        0.28323999 W

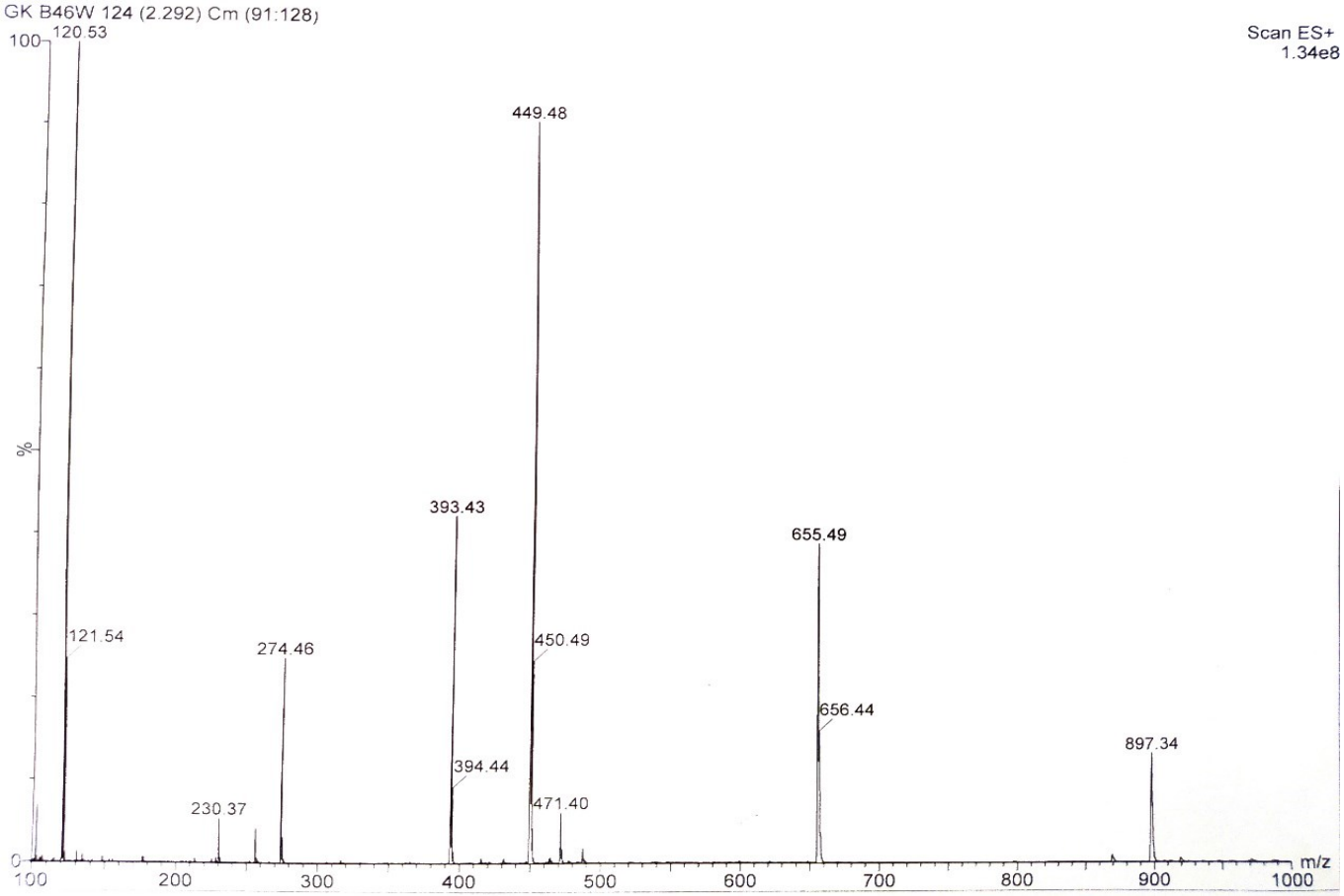
F2 - Processing parameters
SI                32768
SF          242.9370770 MHz
WDW                EM
SSB                   0
LB                 1.00 Hz
GB                    0
PC                 2.00
```

ESI-MS of compound Z-Phe^{P}-(OEt)(OTya-Boc) (**9**).



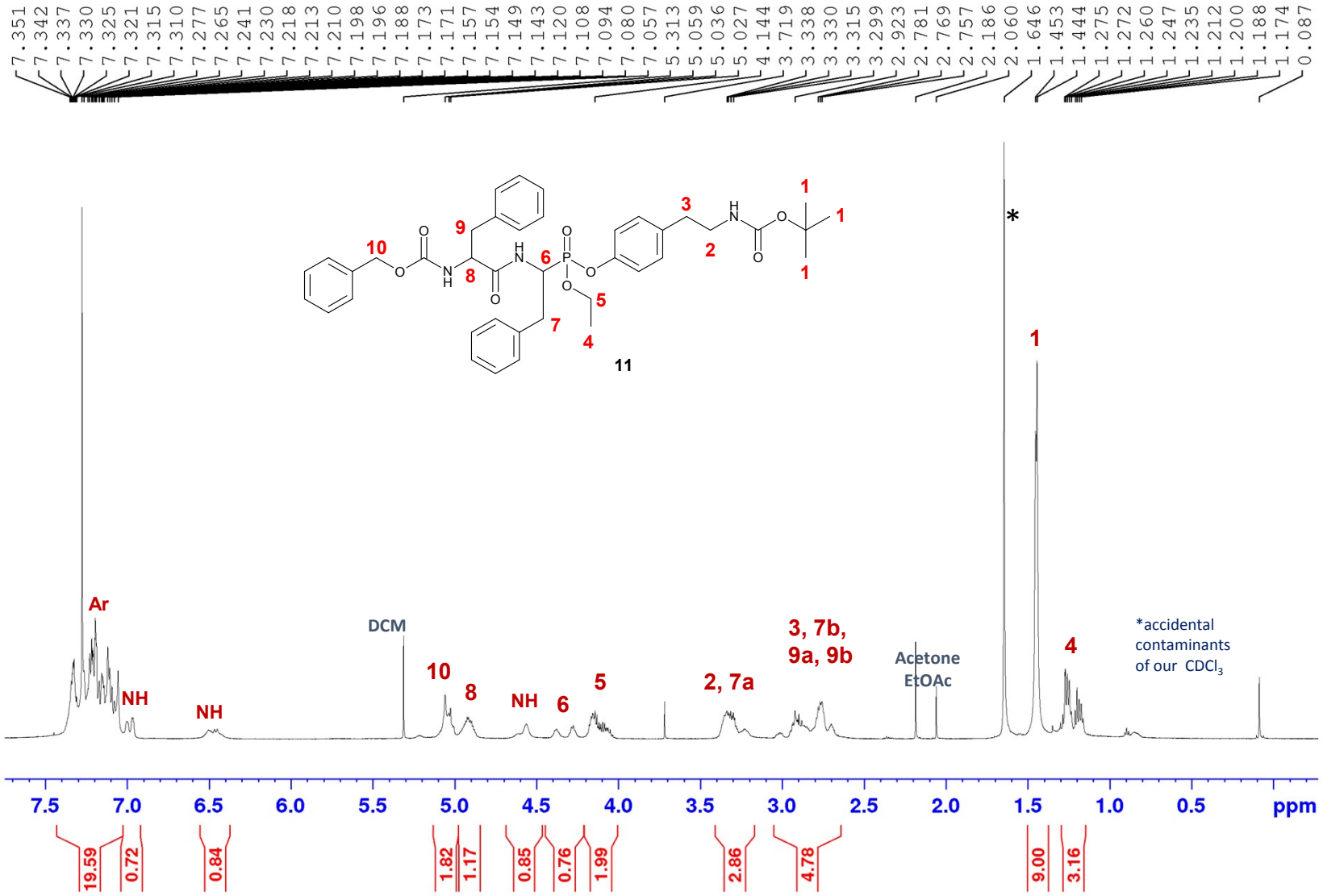
m/z calcd for $[M+Na]^+$ $C_{31}H_{39}N_2NaO_7P^+$ 605.24, found 605.30; calcd for $[M+K]^+$ $C_{31}H_{39}N_2KO_7P^+$ 621.21, found 621.23.

ESI-MS of crude compound H_2N-Phe^P-(OEt)(OTya-Boc) (**10**).

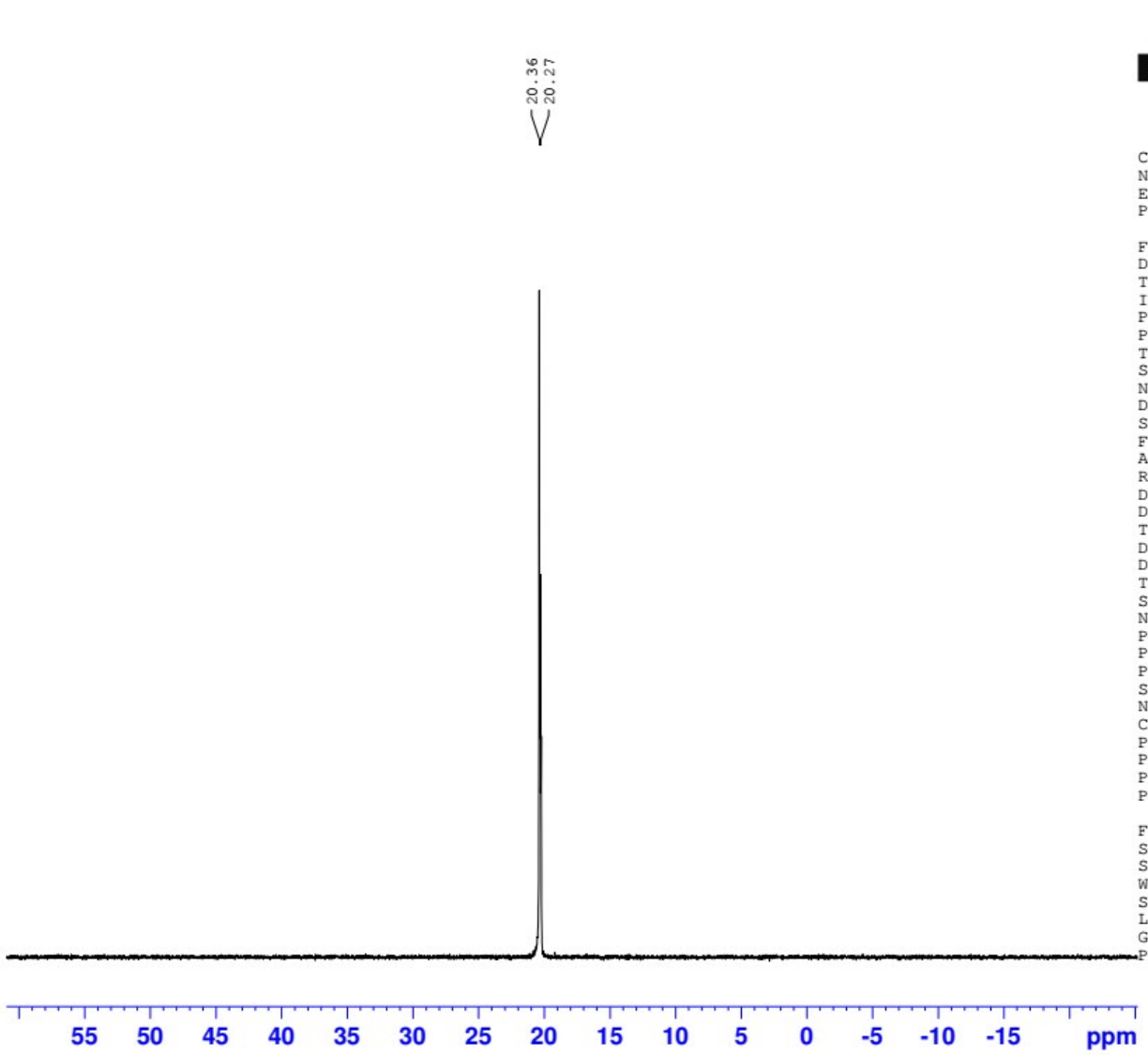


m/z calcd for $[M+H]^+$ $C_{23}H_{34}N_2O_5P^+$ 449.22, found 449.48.

^{1}H NMR (600 MHz, $CDCl_3$) of compound Z-Phe-Phe^{P}-(OEt)(OTya-Boc) (**11**).



^{31}P NMR (243 MHz, $CDCl_3$)of compound Z-Phe-Phe^{P}-(OEt)(OTya-Boc) (**11**).

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Current Data Parameters
NAME      E14c15-20_31P
EXPNO                 2
PROCNO                1

F2 - Acquisition Parameters
Date_          20210114
Time              16.43 h
INSTRUM            spect
PROBHD   Z114607_0214 (
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                  101
DS                    4
SWH           96153.844 Hz
FIDRES         2.934382 Hz
AQ            0.3407872 sec
RG               192.58
DW                5.200 usec
DE                6.50 usec
TE                298.2 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1
SFO1        242.9249301 MHz
NUC1               31P
P0                4.00 usec
P1               12.00 usec
PLW1        36.85100174 W
SFO2        600.1324005 MHz
NUC2               1H
CPDPRG[2        waltz16
PCPD2             70.00 usec
PLW2        27.59199905 W
PLW12        0.56309998 W
PLW13        0.28323999 W

F2 - Processing parameters
SI                32768
SF          242.9370770 MHz
WDW                EM
SSB                   0
LB                1.00 Hz
GB                    0
PC                2.00
```
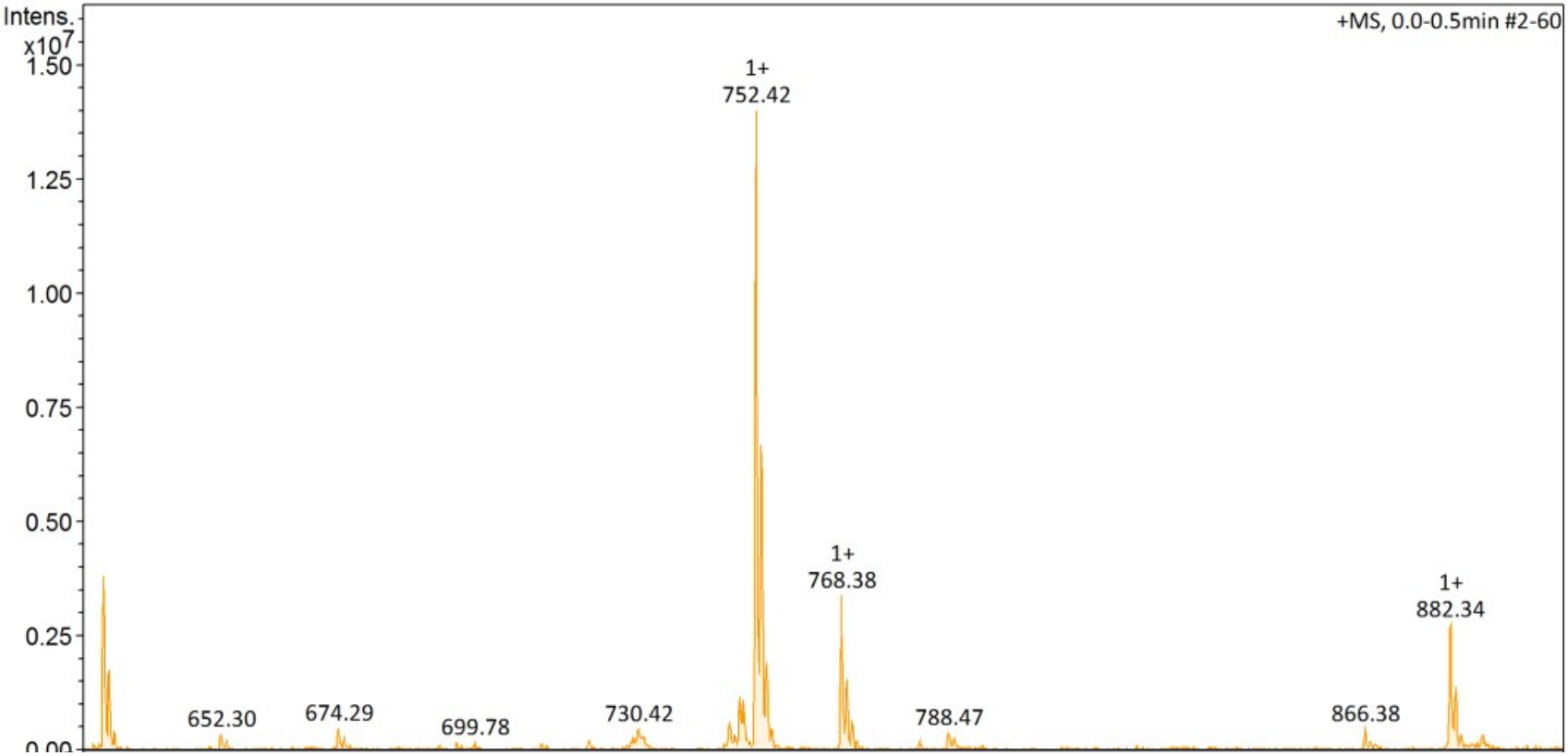
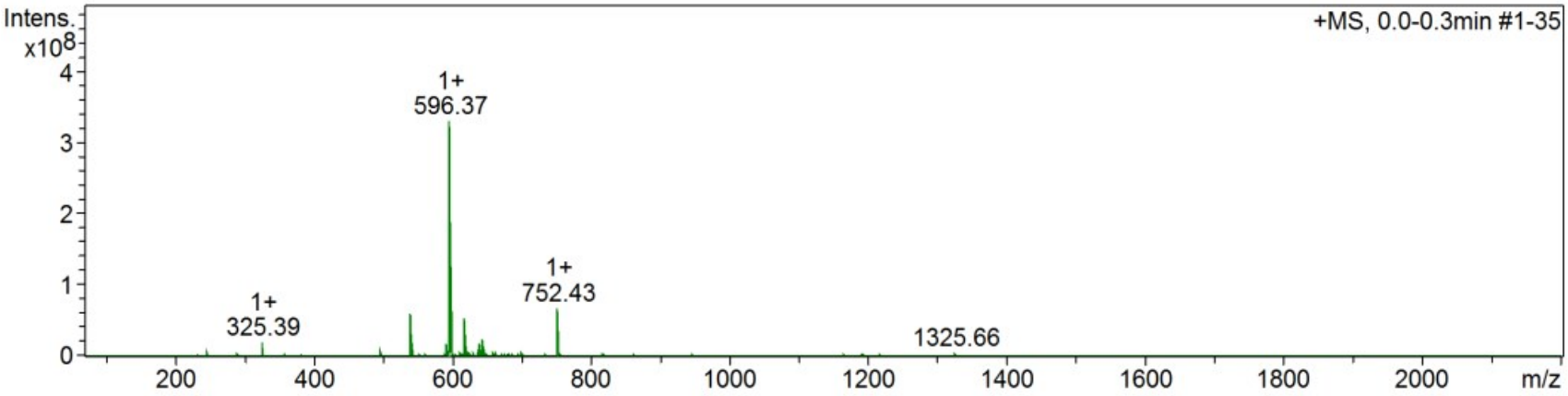

ESI-MS of compound Z-Phe-Phe^{P}-(OEt)(OTya-Boc) (**11**).



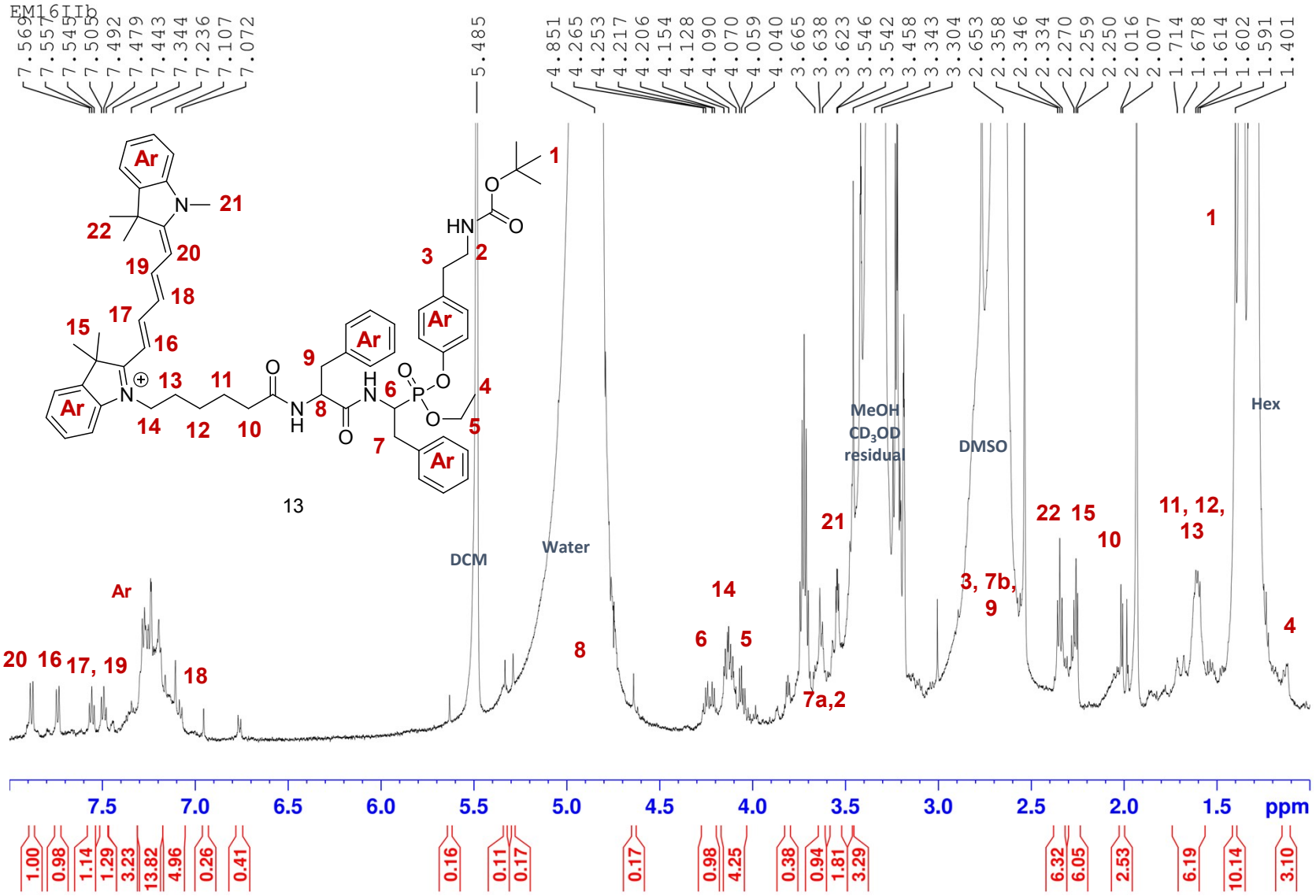
m/z calcd for $[M+Na]^+$ $C_{40}H_{48}N_3NaO_8P^+$ 752.31, found 752.42; calcd for $[M+K]^+$ $C_{40}H_{48}KN_3O_8P^+$ 768.28, found 768.38.

ESI-MS of compound H_2N-Phe-Phe^P-(OEt)(OTya-Boc) (**12**).

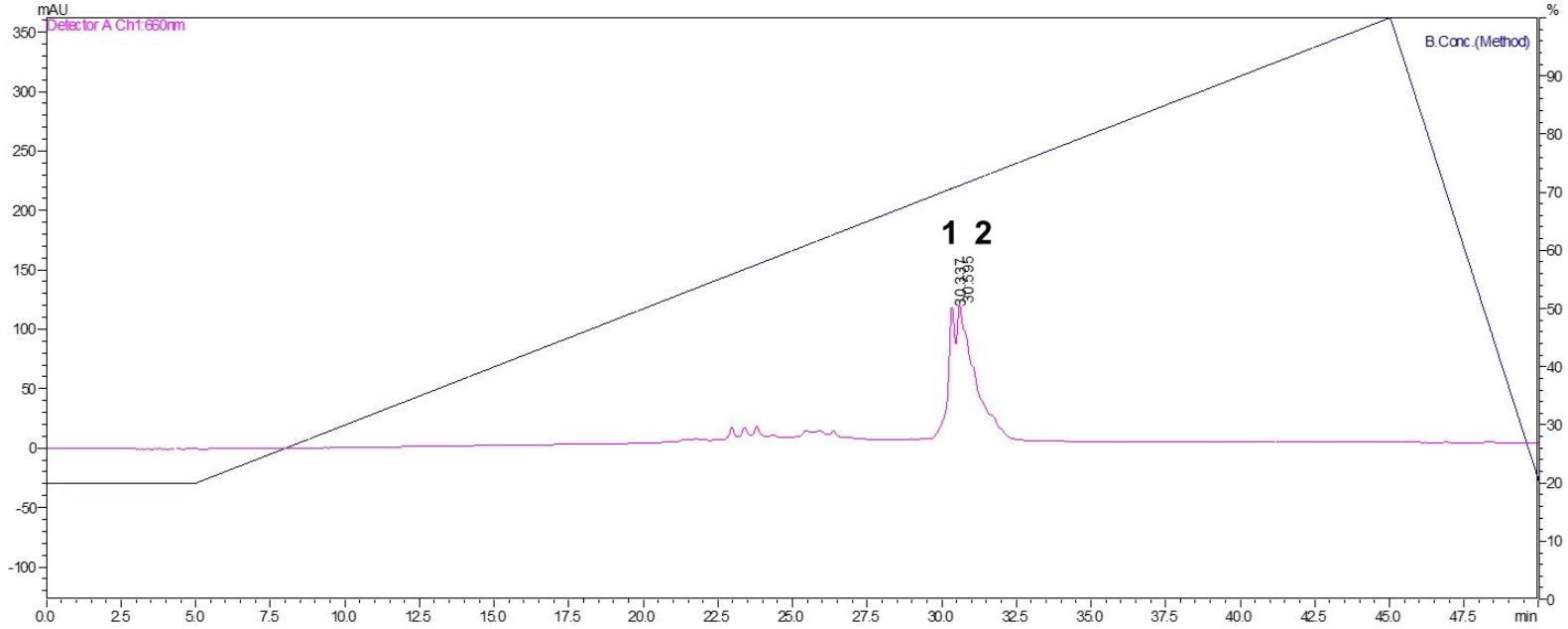


m/z calcd for $[M+H]^+$ $C_{32}H_{43}N_3O_6P^+$ 596.29, found 596.37; calcd for $[M+Na]^+$ $C_{32}H_{42}N_3NaO_6P$ 618.27, found 618.36.

^{1}H NMR (600 MHz, CD_3OD) of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-Boc) (**13**).

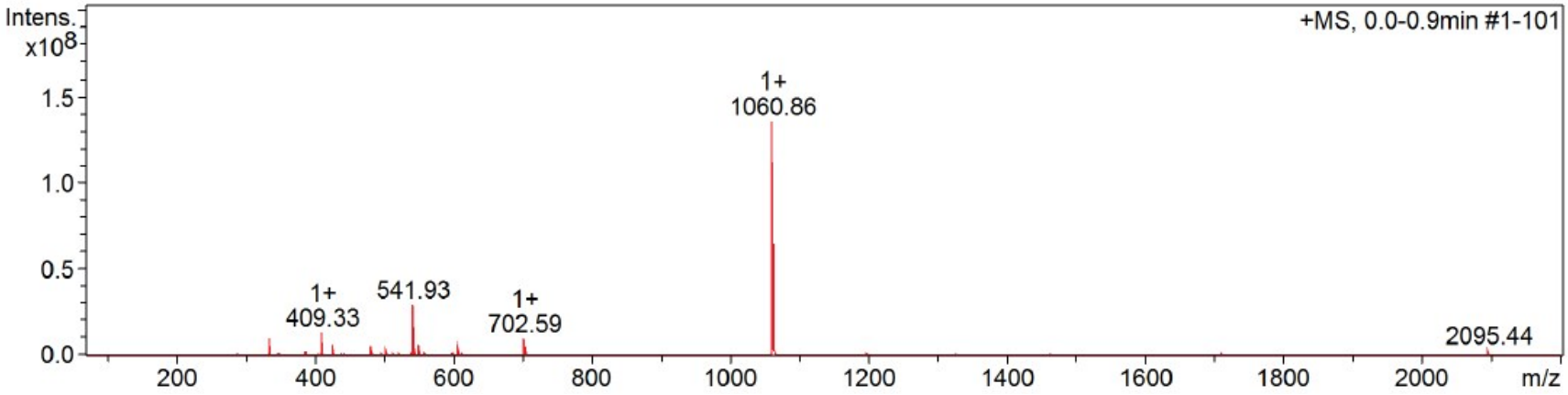


HPLC of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-Boc) (**13**).



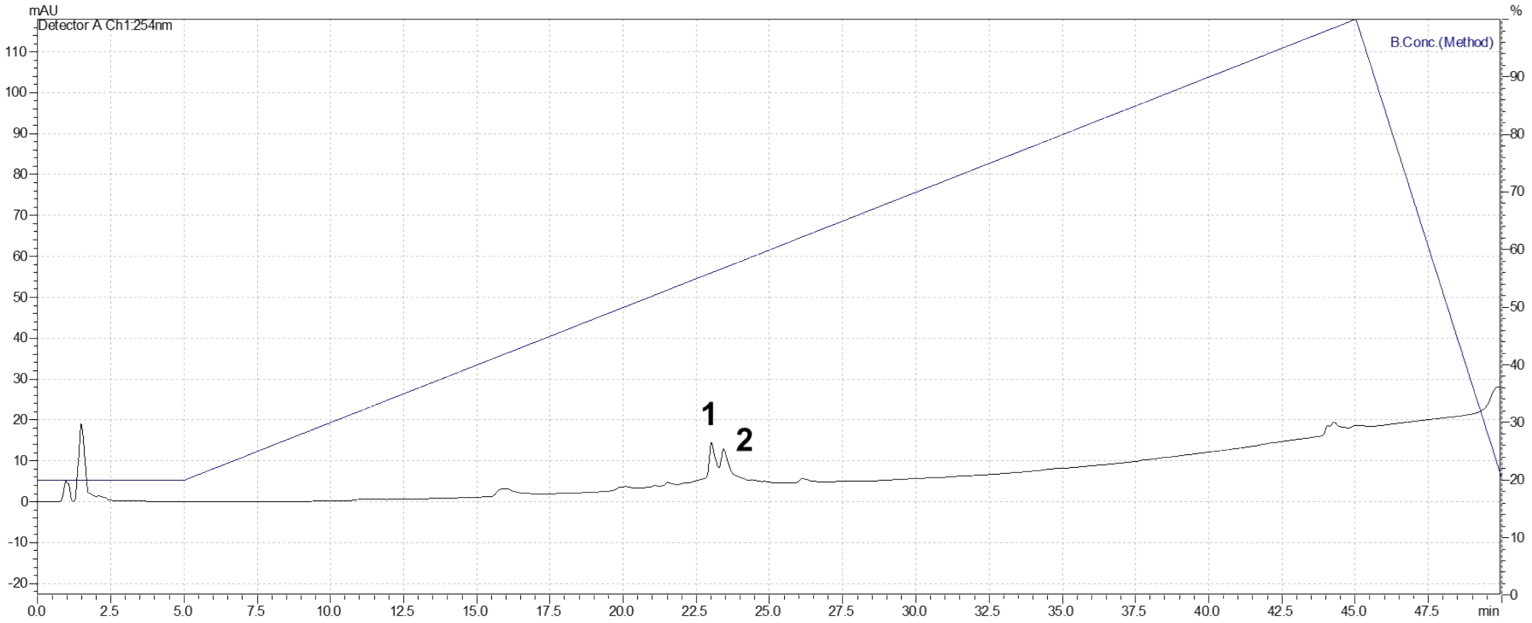
t_R: peak 1: 30.34 min (70.66% B), peak 2: 30.59 min 71.15% (diastereomers)

ESI-MS of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-Boc) (**13**).



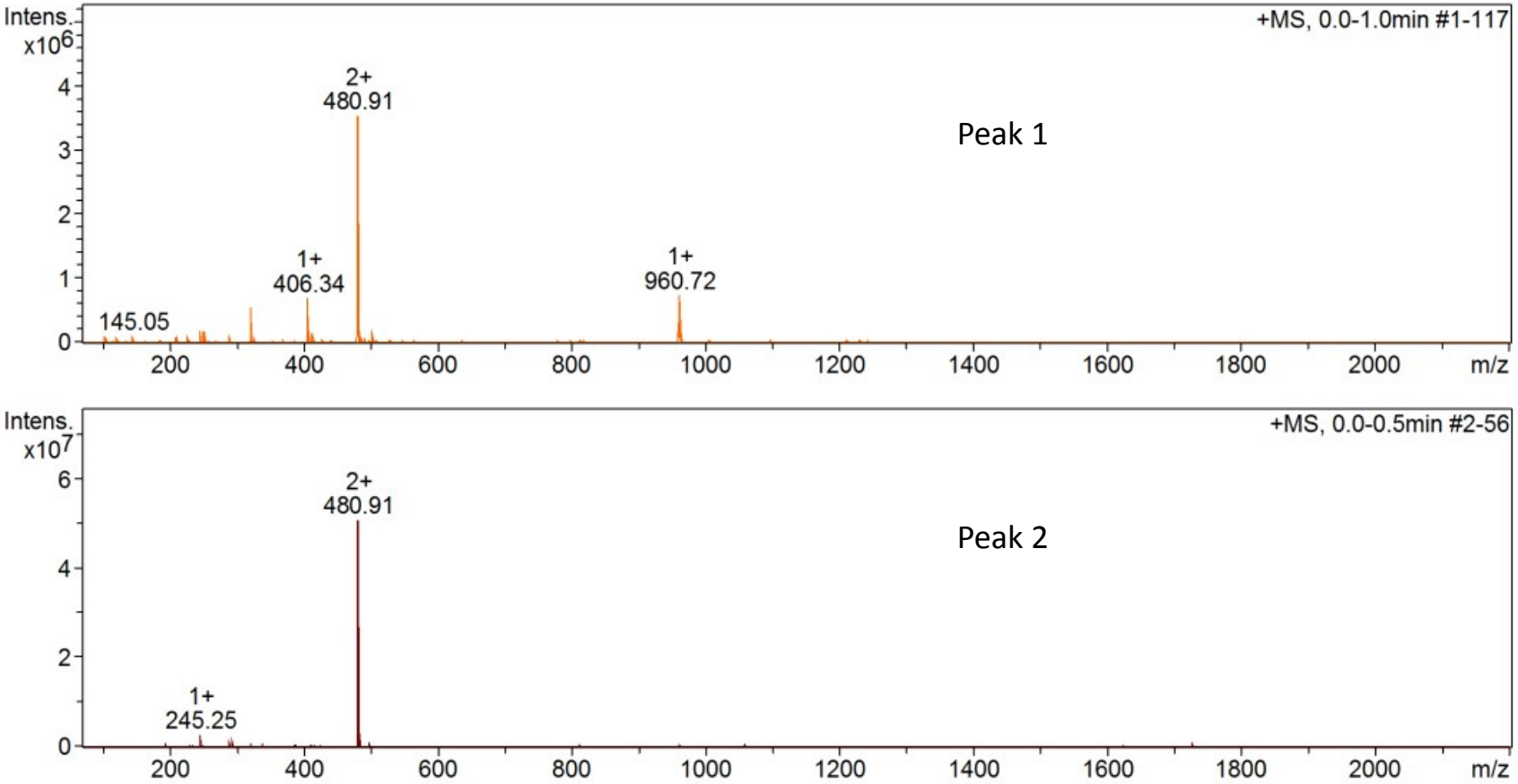
m/z calcd for [M]$^{+}$ $C_{64}H_{79}N_5O_7P^{+}$ 1060.57, found 1060.86; calcd for [M+Na]$^{2+}$/2 $C_{64}H_{79}N_5NaO_7P^{2+}$ 541.78, found 541.93.

HPLC of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-NH_2) (**14**).



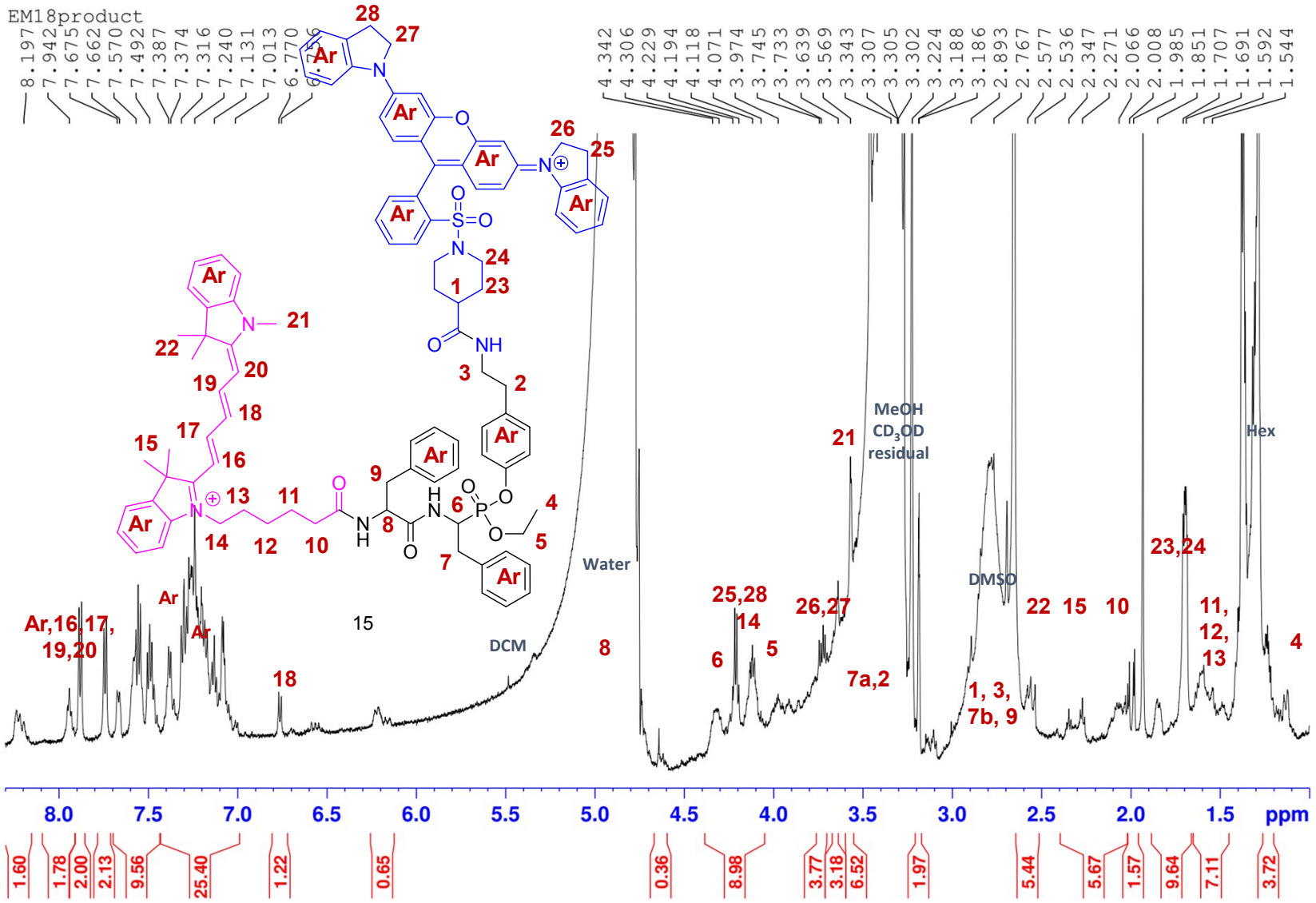
t_R: peak 1: 23.08min (56.10% B); peak 2: 23.45 min (56.57% B) (diastereomers)

ESI-MS of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-NH_2) (**14**).

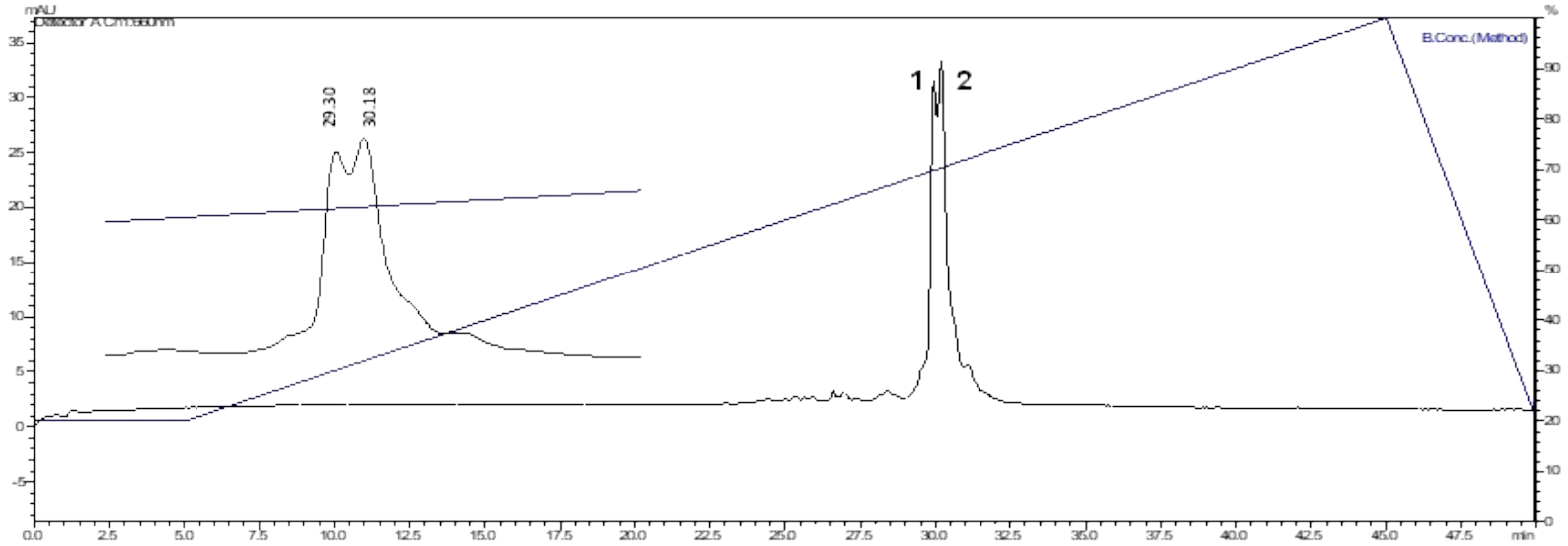


m/z calcd for $[M+H]^{2+}/2$ 480.76, found 480.91; calcd for $[M]^{+}$ $C_{59}H_{71}N_5O_5P^{+}$ 960.52 found 960.72.

^{1}H NMR (600 MHz, CD_3OD) of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-QSY21) (**15**).

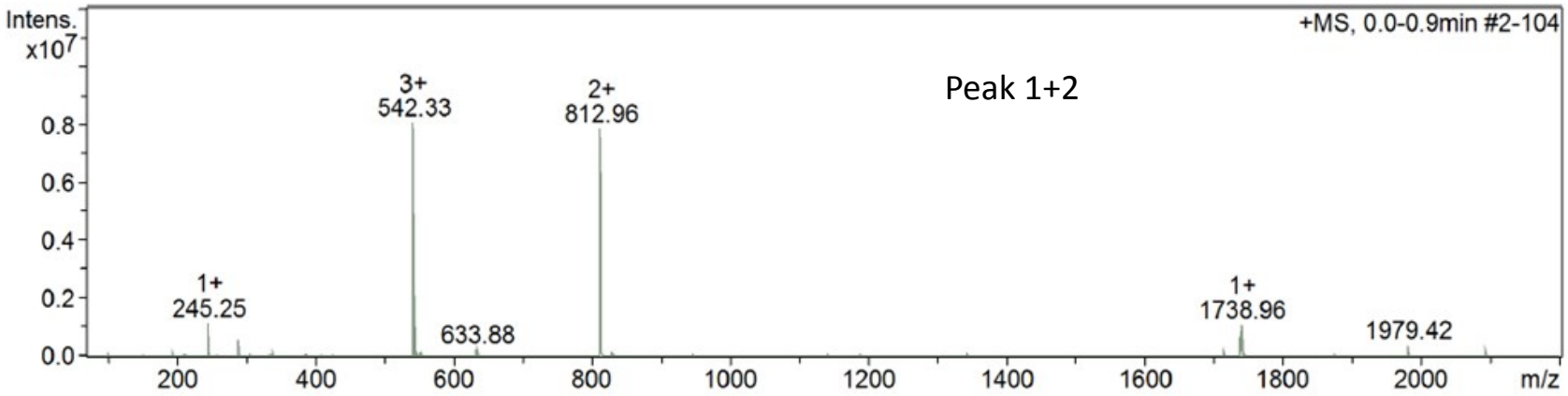


HPLC of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-QSY21) (**15**).



t_R: peak 1: 29.30 min (69.48% B), peak 2: 30.18 min (69.95% B) (diastereomers).

ESI-MS of compound Cy5-Phe-Phe^{P}-(OEt)(OTya-QSY21) (**15**).



m/z calcd for $[M]^{2+}/2$ $C_{100}H_{105}N_8O_9PS^{2+}$ 812.87, found 812.96; calcd for $[M+H]^{3+}/3$ 542.25, found 542.33.