Supplementary Information

Novel Proton Acceptor Immonium-Type Coupling Reagents: Application in Solution and Solid Phase Peptide Synthesis

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Experimental Section

General

TLC was performed on silica plates (8×4 cm) from Albet using suitable solvent systems and visualization by a Spectroline UV Lamp Model CM-10 (254 nm). Melting points were obtained in open capillary tubes using a Buchi melting point B-540 apparatus and uncorrected. Infrared spectra (IR) were recorded on a FT-IR Thermo Nicolet series Fourrier Transformer instrument as KBr pellets. Absorption bands (ν_{max}) were given in wave numbers (cm^{-1}) using UV-Vis Spectrophotometer, Shimadzu UV-250/PC. NMR spectra were recorded on a Varian mercury 400 MHz spectrometer at room temperature. Tetramethylsilane (TMS) was used as reference for all NMR spectra with chemical shifts reported as ppm relative to TMS. HPLC analyses were carried out using Waters Symmetry Column C_{18}, 5μ, 4.6x150mm with dual λ absorbance detector. HPLC-MS analyses were carried out using Waters Symmetry Column C_{18}, 5μ, 4.6x150mm with dual λ absorbance detector, Waters micromass ZQ. All solvents used for recrystallization, extraction, column chromatography and TLC were commercial grade, distilled before use and stored under dry conditions.

N,N-Dimethylmorpholine-4-carboxamide (5)

Morpholine (1 mol) was dissolved in DCM-10% NaOH (1:1, 600 mL) and *N,N*-dimethyl carbamoyl chloride (1.2 mol in 200 ml of DCM) added in 10 min. The reaction mixture was stirred at room temperature for 3 hr and then the organic layer was collected. The NaOH layer was washed with DCM (200 mL) and the combined DCM washed with water (2x100 mL), sat. $NaCO_3$ (2x100 mL), dried ($MgSO_4$) and filtered. Next, the solvent was removed under vacuum to give urea derivatives as pure colorless oil (138 g, 87.3%) as observed by NMR spectra. ^{1}H NMR ($CDCl_3$): δ 2.84 (s, 6H, 2 CH_3), 3.22-3.28 (m, 4H, 2CH_2), 3.68-3.70(m, 4H, 2CH_2) ppm.^{13}C NMR ($CDCl_3$): δ 38.62, 47.51, 66.89, 164.96 ppm.

Synthesis of *4-((dimethyamino)chloromethylene)morpholin-4-Imminium Hexaflurophosphate (DCMH, 6)*[10]

Oxalyl chloride (100 mmol) in dry DCM (100 mL) was added dropwise to a solution of **5** (100 mmol) in dry DCM (200 mL) at room temperature over a period of 5 minutes. After the reaction mixture was stirred under reflux for 3 hours, the solvent was removed under vacuum and the residue washed with anhydrous diethylether (2 x 100 mL). N_2 was then bubbled to remove excess ether. The white solid obtained was dissolved in DCM (500 mL), and a saturated aqueous KPF_6 solution (18.4 g in 50 mL water) was added at room temperature stirring vigorously for 10-15 minutes. The organic layer was collected, washed once with H_2O (50 ml), dried over anhydrous $MgSO_4$, and filtered. The solvent was then removed under reduced pressure to give a white solid which recrystallized from DCM-ether to give white crystals in yield 89.6% (28.9 g), mp 94-95°C. ^{1}H NMR (CD_3COCD_3): δ 3.39 (s, 6H, 2CH_3), 3.75(t, 4H, 2CH_2), 3.86 (t, 4H, 2CH_2) ppm. ^{13} C NMR (CD_3COCD_3): δ 44.36, 52.82, 65.99, 162.79 ppm.

Elemental analysis Calcd for $C_7H_{14}ClF_6N_2OP$ (322.5): C; 26.05, H; 4.34; N; 8.68.

Found: C; 25.94, H; 4.43; N; 8.79.

General Method for Synthesis of Immonium Type Coupling Reagents

6 (6.45 g) was added to a solution of HOXt (20 mmol) and Et_3N (20 mmol, 2.8g) in DCM (50 mL) or KOXt (20 mmol) in acetonitrile (40 mL) at 0 ^{o}C. The reaction mixture was stirred at this temperature and left at room temperature overnight. Next, the mixture

was filtered and washed with cooled DCM. The white solid was recrystallized from acetonitrile-diethylether

***Synthesis 1-((Dimethylimino)(morpholino)methyl)3-H-[1,2,3]triazolo[4,5-b]pyridine-1-3-olate Hexafluorophosphate.* (HDMA, 7a)**

The product was obtained as white solid in yield 91.2 % (7.68 g), mp.194-195 ^{o}C (dec).

^{1}H NMR (CD_3COCD_3): δ 3.27(s, 3H, CH_3), 3.64(s, 3H, CH_3), 3.85-3.89(m, 4H, $2CH_2$), 4.00-4.07 (m, 4H, $2CH_2$), 7.93-7.96 (dd, 1H, ar), 8.44-8.47 (dd,1H, ar), 8.76-8.77 (dd, 1H, ar) ppm. ^{13}C NMR (CD_3COCD_3): δ 41.74, 42.35, 50.82, 51.58, 66.19, 66.42, 124.37, 127.84, 149.65 ppm. m/z using MALDI with ACH matrix 422.2.

Elemental analysis Calcd for $C_{12}H_{17}F_6N_6O_2P$ (422): C; 34.13, H; 4.06; N; 19.90.

Found: C; 34.31, H; 4.17; N; 20.13.

Synthesis *1-((dimethylimino)(morpholino)methyl)3-H-benzo[1,2,3]triazolo-1-ium-3-olate Hexafluorophosphate.* (HDMB, 7b)

The product was obtained as white solid in yield 88.3 % (7.54 g), mp.196-197 ^{o}C (dec).

^{1}H NMR (CD_3COCD_3): δ 3.22(s, 3H, CH_3), 3.51(s, 3H, CH_3), 3.66-3.88(m, 4H, $2CH_2$), 4.03-4.06 (m, 4H, $2CH_2$), 7.65-7.96 (dt, 1H, ar), 7.86-7.92 (dm,2H, ar), 7.98.7.99 (dd, 1H, ar) ppm.

^{13}C NMR (CD_3COCD_3): δ 41.76, 42.23, 50.81, 51.51, 66.19, 66.41, 109.99, 114.56, 127.52, 133.54 ppm. m/z using MALDI with ACH matrix 421.23.

Elemental analysis Calcd for $C_{13}H_{18}F_6N_5O_2P$ (421): C; 37.06, H; 4.31; N; 16.62.

Found: C; 36.98, H; 4.26; N; 16.73.

Synthesis of 1-((dimethylimino)(morpholino)methyl)3-H-6-chlorobenzo[1,2,3] triazolo -1-ium-3-olate Hexafluorophosphate. (6-HDMCB, 7c)

The product was obtained as white solid in yield 93.5%, mp 193-194 ^{o}C (dec)

^{1}H NMR (CD_3COCD_3): δ 3.31(s, 3H, CH_3), 3.69(s, 3H, CH_3), 3.94-3.951(m, 4H, $2CH_2$), 4.12-4.14 (m, 4H, $2CH_2$), 7.96-8.03 (qd, 2H, ar), 8.12-8.13 (dd,1H, ar) ppm.

^{13}C NMR (CD_3COCD_3): δ 41.78, 42.30, 50.84, 51.58, 66.21, 66.39, 115.77, 116.18, 132.85, 133.93, 150.49 ppm. Elemental analysis Calcd for $C_{13}H_{17}ClF_6N_5O_2P$ (455.5): C; 34.25, H; 3.73; N; 15.37. Found: C; 34.39, H; 3.83; N; 15.54.

Solubility experiments. DMF solutions (0.5 M, 0.75 M, and 1 M) of 7a, 7b, 7c, HATU, and HBTU were prepared.

Model Segment Coupling Reaction. Test couplings were carried out as previously described for Fmoc-Val-Val-NH_2, Z-Phg-Pro-NH_2, and Z-Phe-Val-Pro-NH_2.[3,12]

Determination of the extent of coupling of Fmoc-Val-OH with H-Val-NH_2 in solution phase by using HPLC analysis.

The above reaction was repeated using different coupling reagents and the samples (10 μL) taken at different times. The reaction was quenched by adding acetonitrile-H_2O (1:1, 2 mL), and 10 μL was then injected to the HPLC under the following condition:

UV detection was carried out at 220 nm, and linear gradients of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA) from 10% to 90% were run at a 1.0 mL·min^{-1} flow rate over 30 min.

Column: Waters C_{18}, 5μm, 4.6x150mm. with dual wavelength detector.

t_R= 20.89 min (Fmoc-Val-OH), t_R = 19.88 min (Fmoc-Val-Val-NH_2) .

Synthesis of H-Tyr-Gly-Gly-Phe-Leu- NH_2 in solution phase

The pentapeptide was synthesized using Boc-chemistry in solution phase except for the last amino acid that Fmoc-Tyr(OtBu)OH was used. HDMB (**7b**) (0.05 mmol) was added to a solution of H-Leu.NH_2 (0.05 mmol), DIEA (0.1 mml), Boc-Phe-OH (0.05 mmol) in DMF (2 mL) at 0 ^oC. The reaction mixture was stirred at 0 ^oC for 1h and at room temperature for another 2h, then ethylacetate was added (20 mL). The organic layer was washed with sat. Na_2CO_3 (2x10 mL), 10% citric acid (2x10 mL) and sat. NaCl (2x10), and then dried ($MgSO_4$) and filtered. Next, the solvent was removed under vacuum. The crude dipeptide was treated by TFA-DCM (1:1, 10 mL) at room temperature for 2h,. The solvent and TFA were then removed under vacuum and washed with diethylether. The crude TFA salt was used for the next step for coupling with Boc.Gly-OH using the same previous coupling techniques. This was followed by the usual work-up. The crude tripeptide was injected to the HPLC using UV detection set at 220 nm. Linear gradients of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA) from 20% to 80%, were run at a 1.0 mL·min^{-1} flow rate over 30 min.

Column: Waters C_{18}, 5μm, 4.6x150mm. with dual wavelength detector. The purity of the tripeptide at t_R 14.10 min. was 93.3 with MS 434, as observed in the HPLC-MS. Extra peak was observed at t_R 14.85 min, which corresponded to excess Boc-Gly-OH.

The crude tripeptide was used directly in the next step with TFA-DCM and then for coupling with Boc-Gly-OH, as mentioned before. The purity of the tetrapeptide TFA·GGFL-NH_2, as tested by HPLC, using linear gradient from 10% to 90% in 30 min, was 94.0% and the HPLC-MS of the TFA.Gly-Gly-Phe-Leu-NH_2 showed the right mass at 392.3 (none of the smaller peptides were detected).

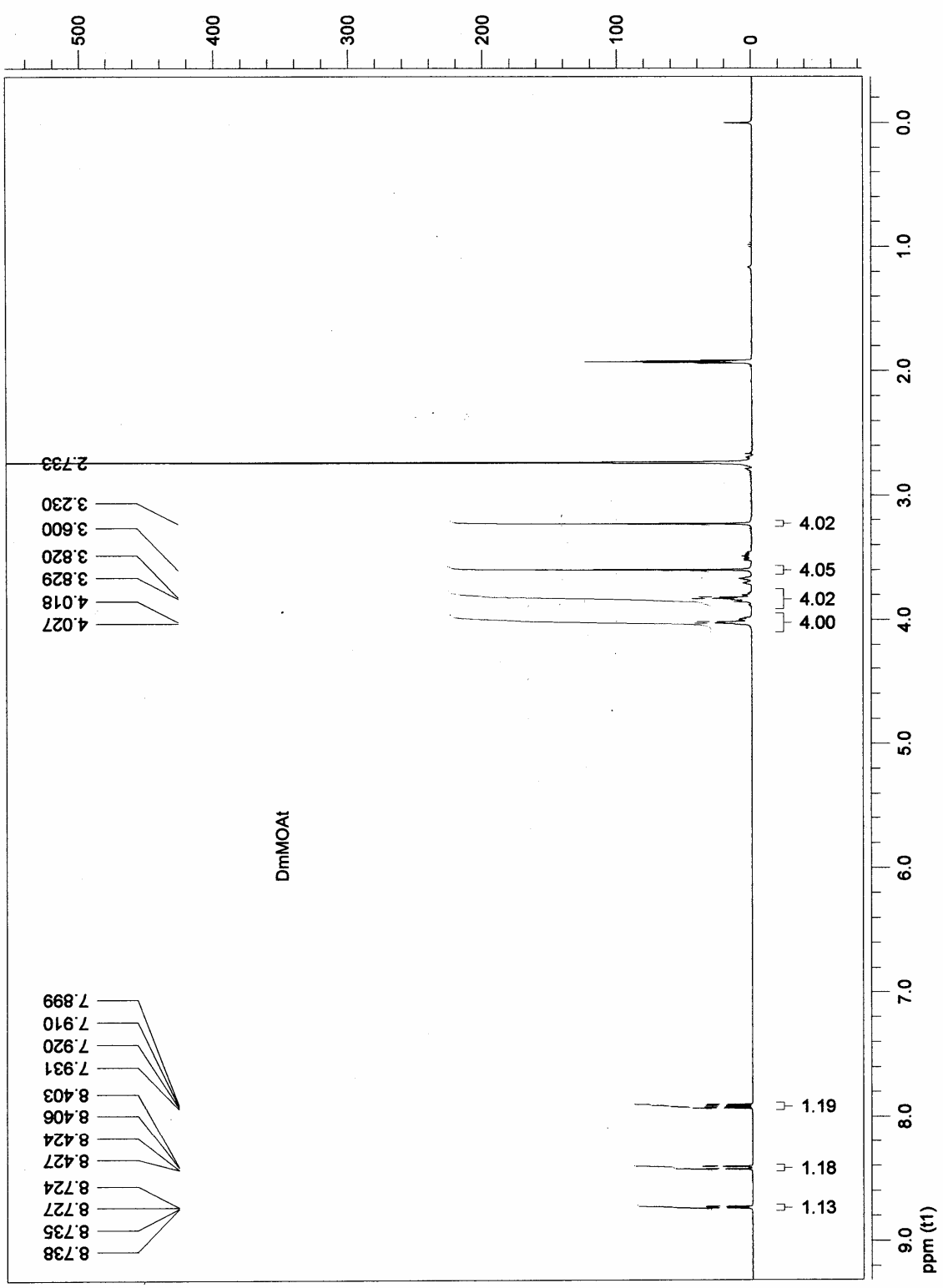
The TFA salt of the tetrapeptide was used directly without purification to couple with Fmoc-Tyr(OBu)-OH under the same condition used before. The Fmoc-group was then removed by treatment with 30% *N,N*-diethylamine in acetonitrile (10 mL) at room temperature for 1h. The solvent was removed under vacuum and then the crude product was treated by 10 mL of mixture of TFA-DCM (1:1) at room temperature for 2h. The solvent and excess TFA were removed under vacuum and then the crude pentapeptide washed with ether to give a slightly brown solid in a 68.9% yield. The purity of the pentapeptide was 98.3 % (at t_R 9.62 min) as tested by HPLC (from 10% to 90% in 30 min). The HPLC-MS showed the right ms for the pentapeptide: *m/z* calcd for $C_{26}H_{25}NO_4S$, 554.3; found, 555.3 $[M + H]^+$.

Solid Phase Synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2 Using Uronium Salts[7]

The pentapeptide was manually assembled on an Fmoc-Rink-Amide-resin (0.63 mmol/g) with preactivation, using Fmoc-amino acid (3 equiv), coupling reagent (3 equiv) and DIEA (6 equiv) in DMF at a total concentration of 0.3 M. A coupling time of 30 min was used for all amino acids, except Aib-Aib, for which the time was 60 min. The peptide was cleaved from the resin using TFA–H_2O (9:1) at room temperature for 2 h. The solution was filtered and the TFA removed under vacuum. The crude peptide was precipitated by adding cold ether, and run on HPLC [UV detection was at 220 nm, and linear gradients of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA) from 10% to 90%, were run at a 1.0 $mL \cdot min^{-1}$ flow rate over 25 min.] Column: Waters C_{18}, 5μm, 4.6x150mm. with dual wavelength detector. The t_R for the pentapeptide and for the des-Aib tetrapeptide was 8.80 min and 9.10 min, respectively.

HDMA (**7a**)	HATU	HDMB (**7b**)	HBTU	6-HDMCB (**7c**)

HPLC traces of H-Try-Aib-Aib-Phe-Leu-NH_2 *vs* H-Try-Aib-Phe-Leu-NH_2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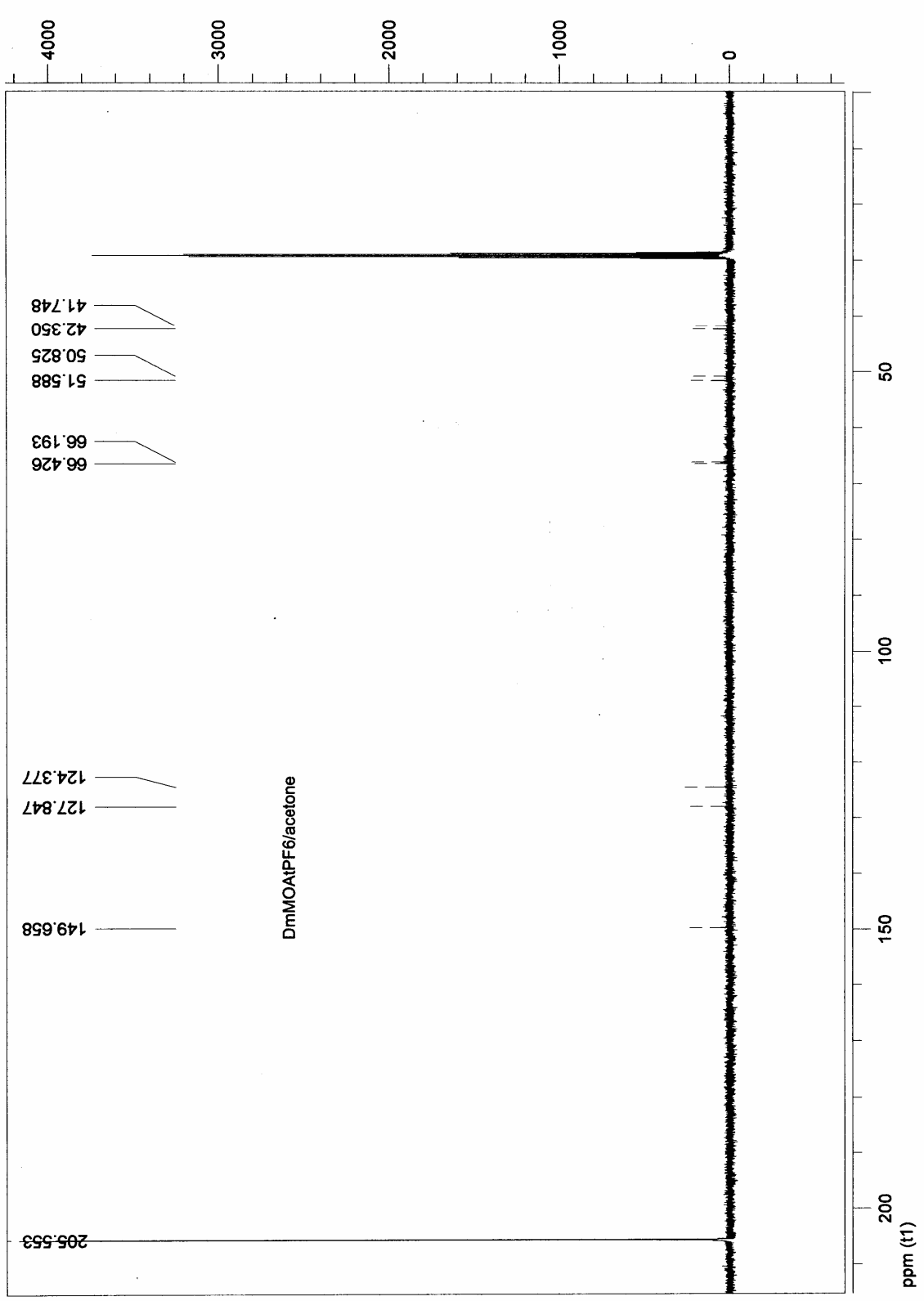
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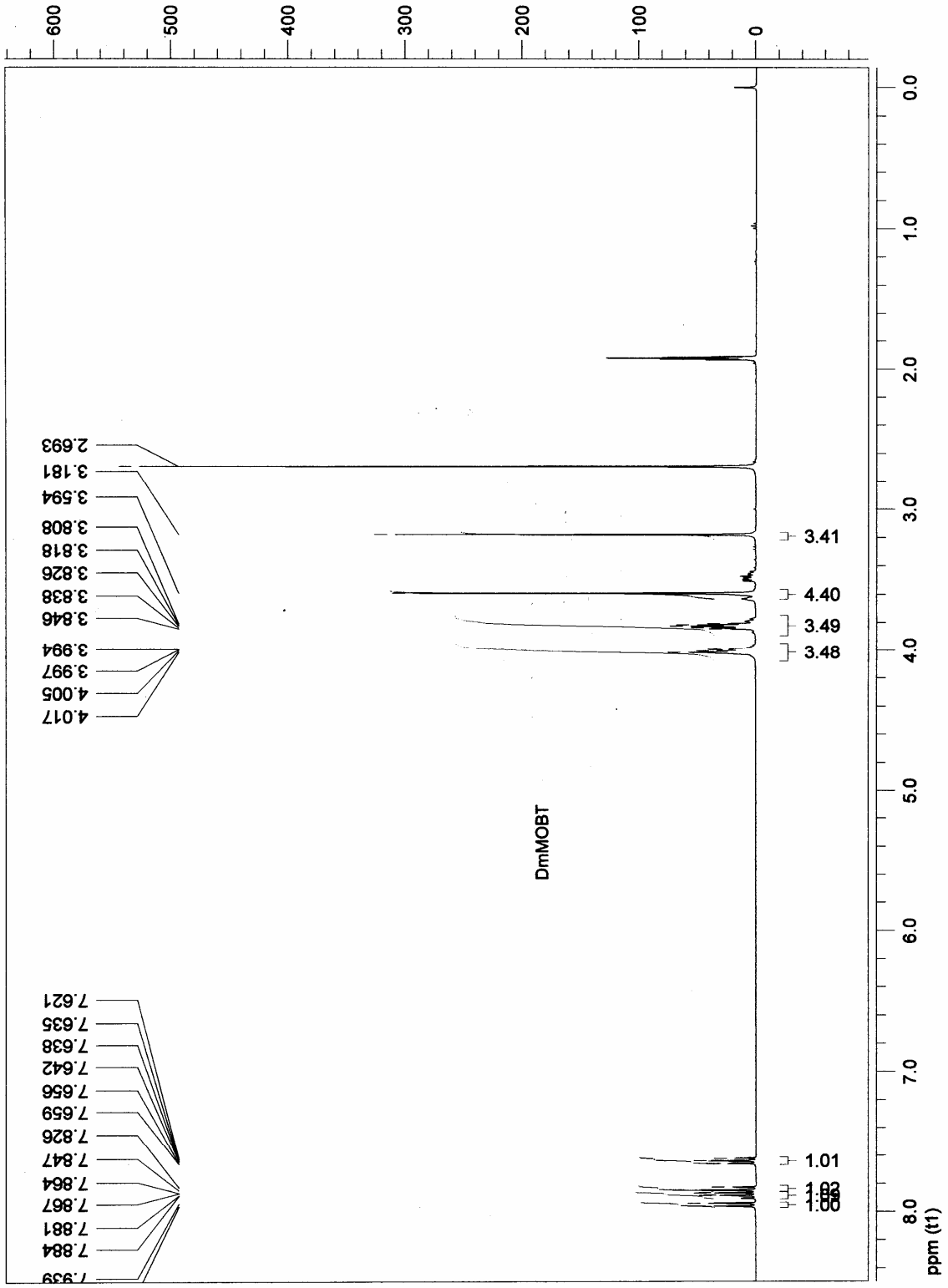
150
100
50
ppm (t1)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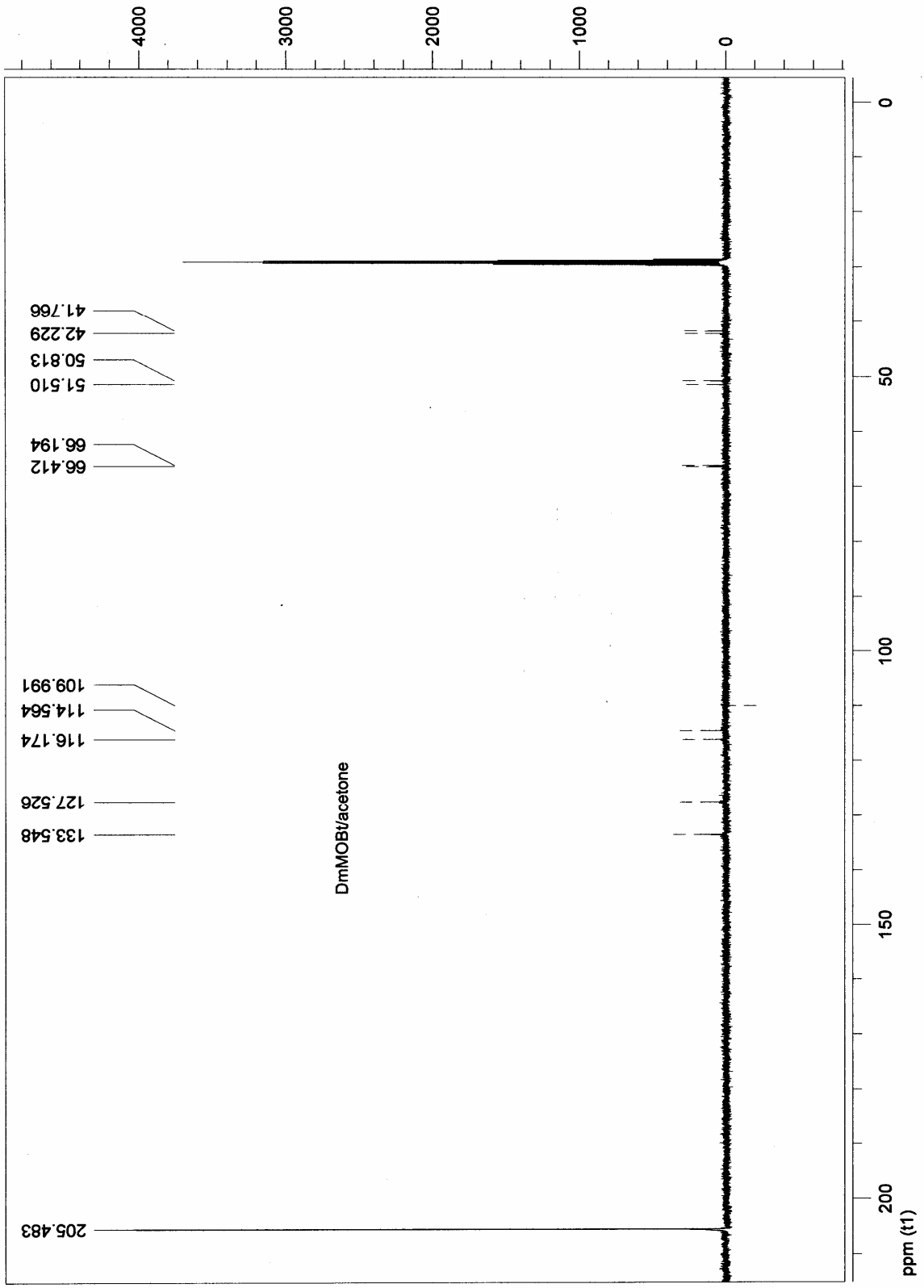
ppm (t1)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2000
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200
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50
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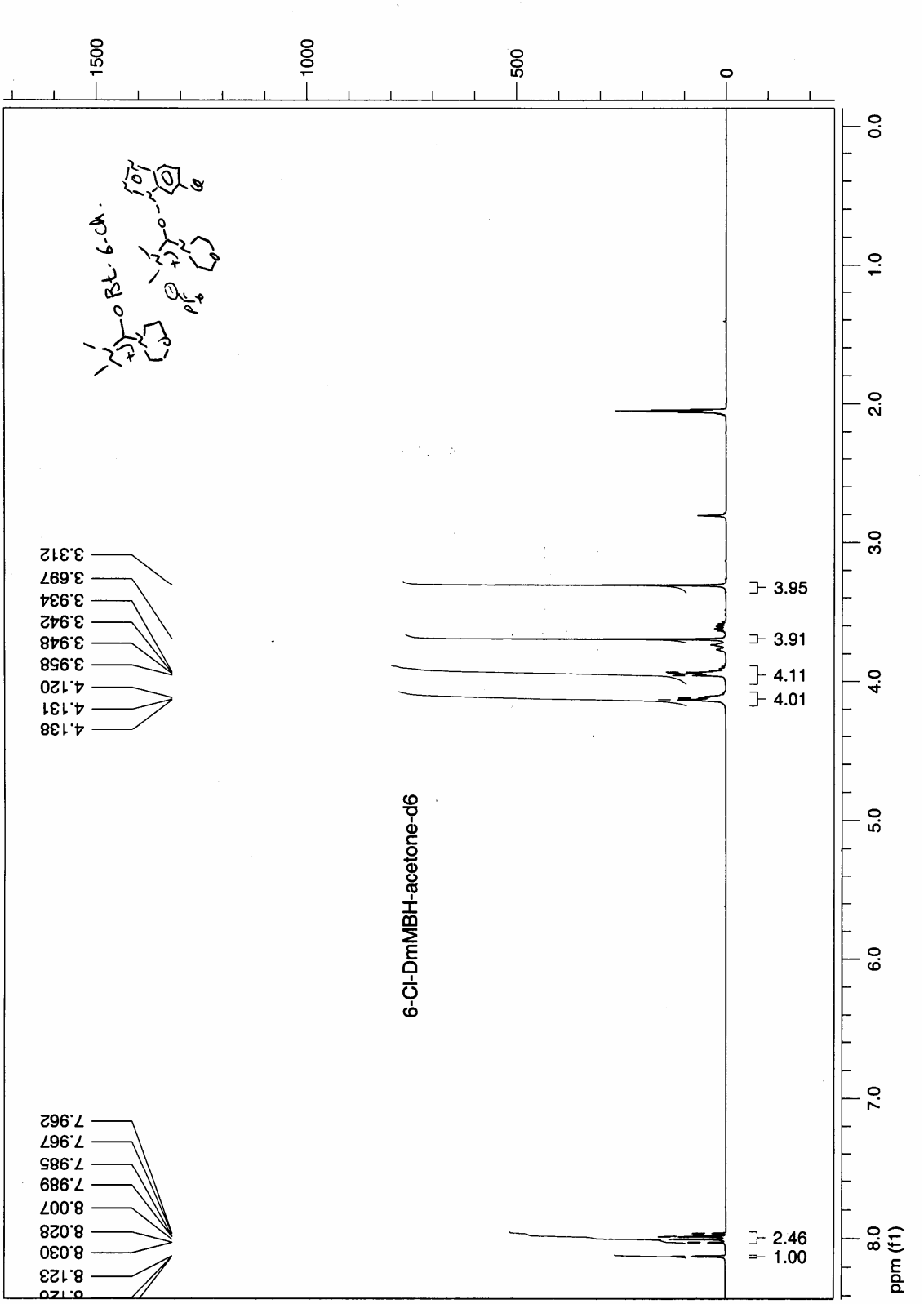
ppm (t1)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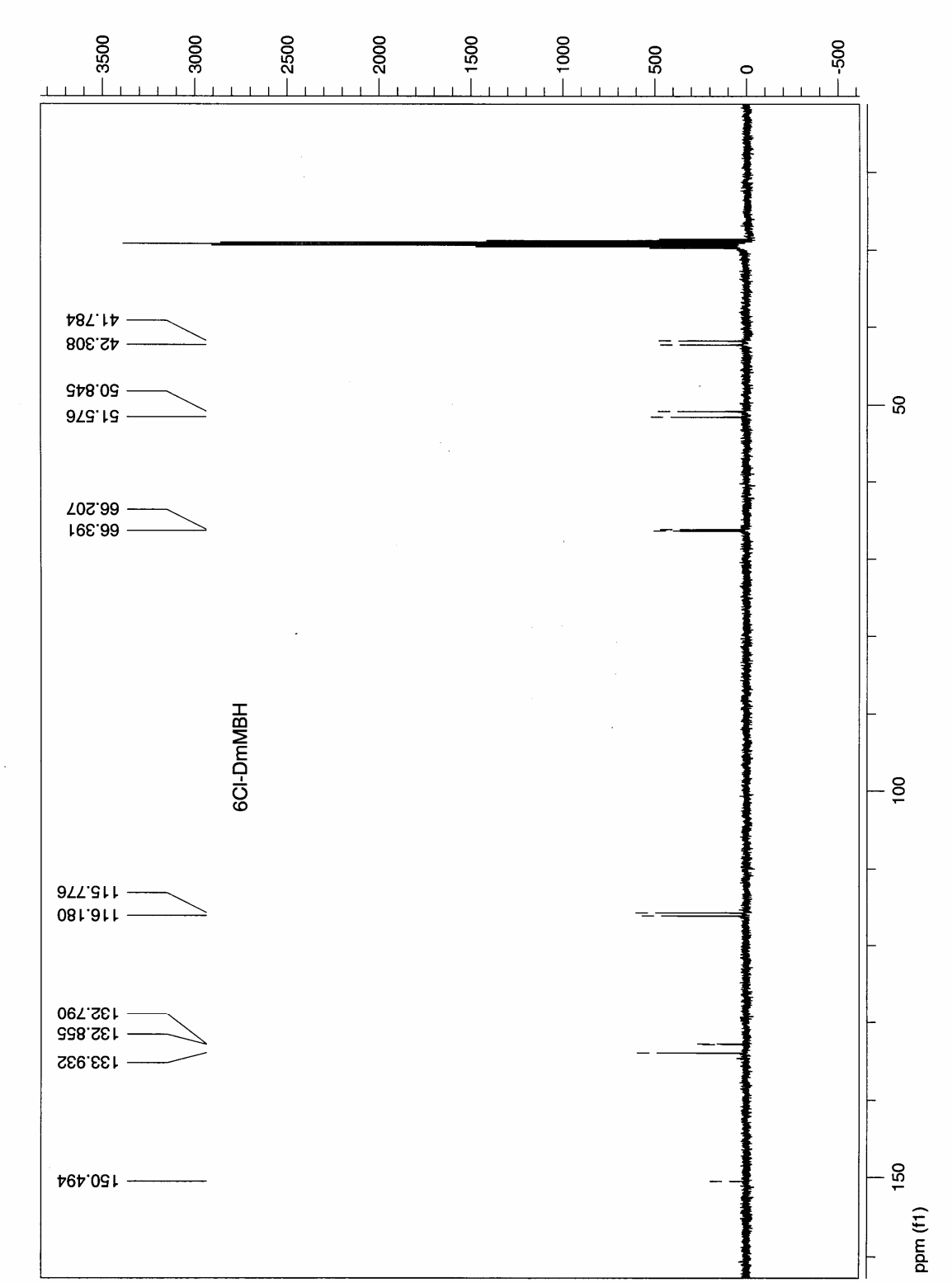
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3000
2500
2000
1500
1000
500
0
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150
100
50
ppm (f1)