Electronic Supplementary Material (ESI) for RSC Advances.

Supporting information

Rapid synthesis of internal peptidyl α-ketoamides by on resin oxidation for the construction of rhomboid protease inhibitors

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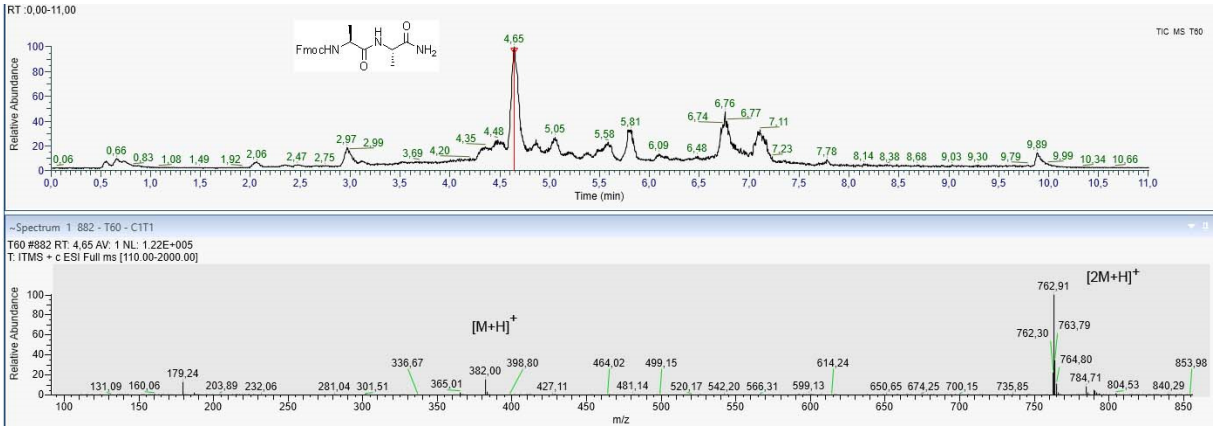


Figure S1. CO extrusion after solid phase synthesis starting from resin-bound intermediate compound **14**, yielding Fmoc-Ala-Ala-NH_2 dipeptide **15**. MS-analysis shows almost clean formation of the dipeptide yielded by CO elimination.

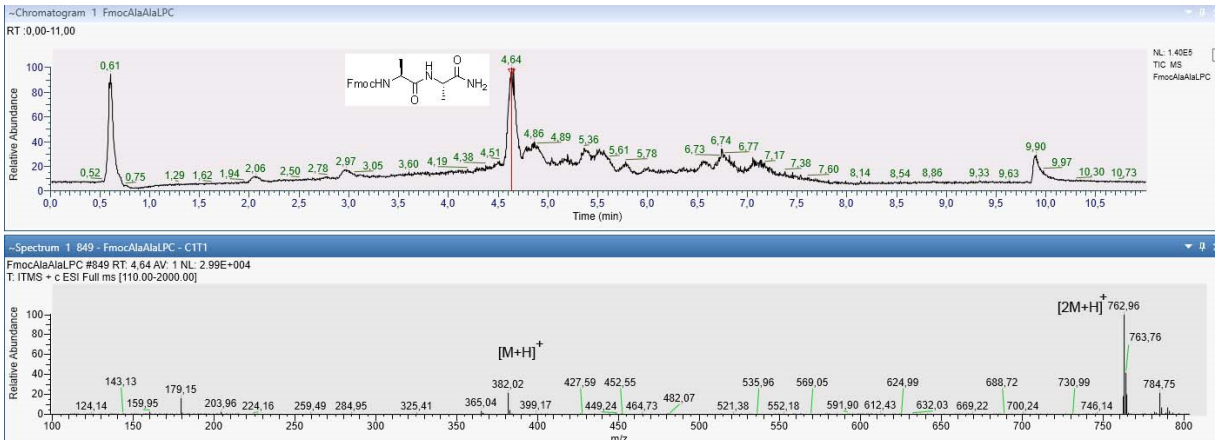


Figure S2. CO extrusion after solution phase coupling starting from alanine amide **16** and ketoacid **13**, yielding Fmoc-Ala-Ala-NH_2 dipeptide **15**.

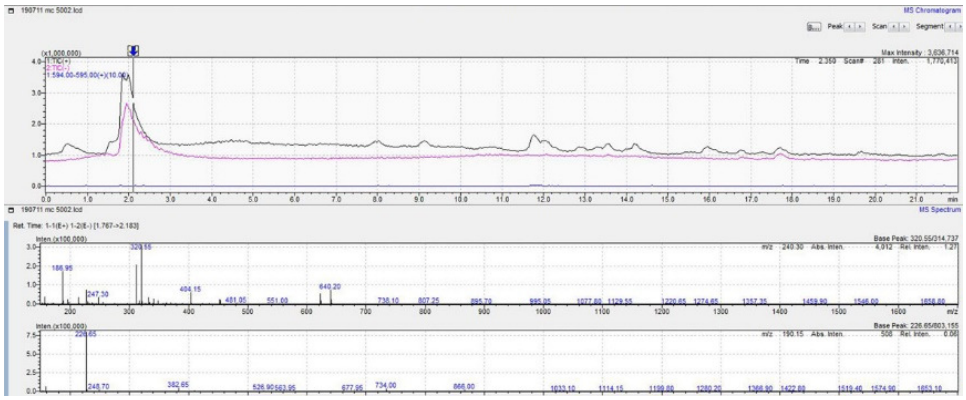


Figure S3. LC-MS of crude compound **18** shows product (622.15 and its hydrate 640.20), but no CO extrusion was observed (extracted ion chromatogram in blue).

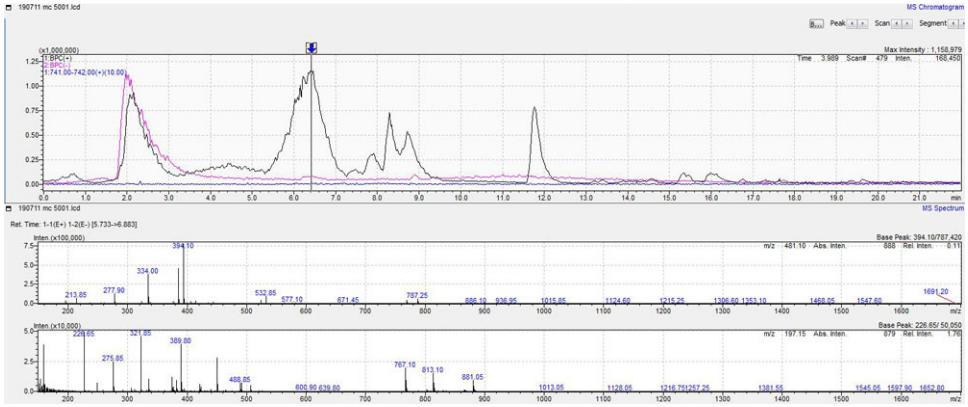


Figure S4. LC-MS of crude compound **19** shows product (769.25 and its hydrate 787.25), but no CO extrusion was observed (extracted ion chromatogram in blue).

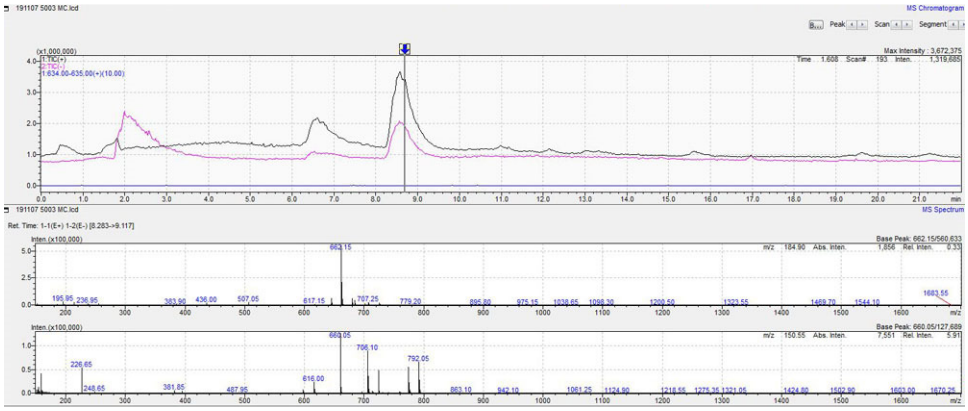


Figure S5. LC-MS of crude compound **20** shows product (662.15) but no CO extrusion was observed (extracted ion chromatogram in blue).

Experimental methods

General materials & equipment

All materials were purchased from commercial vendors and were used as received. TLC analysis was performed on pre-coated ALUGRAM SIL G plates (Carl Roth) with detection by a handheld UV lamp (254 nm) and subsequent staining with cerium ammonium molybdate solution followed by heating. LC-MS analysis was perfomed on a Thermo LCQ Fleet HPLC-MS/MS system or a Prominence Ultra-fast Liquid Chromatography system coupled to a MS-2020 single quadrupole mass analyzer (Shimadzu). HPLC analysis was performed on a Shimadzu Prominence HPLC system equipped with a Waters xBridge C18 (5 µm; 10 x 150 mm) column with a linear gradient of acetonitrile in water with 0.1% trifluoroacetic acid. NMR spectra were recorded on a Bruker UltraShield 300 MHz, 500MHz or 600MHz NMR Spectrometer. Silica column chromatography was performed using 230-400 mesh silica (Kieselgel 60).

N-(9-fluorenylmethoxycarbonyl)-L-alanine *N'*-methoxy-*N'*-methylamide (9)

Fmoc-L-alanine monohydrate (1 eq.) and HOBt.H_2O (1.5 eq.) were dried by co-evaporation of water with dry toluene (three times). The mixture was then dissolved in DCM/DMF (8/1; 5 mL per mmol of alanine) and stirred in an icebath for 10 min to allow to cool to 0 °C. Next, (*N*,*O*-dimethylhydroxylamine*HCl (1.5 eq.), HBTU (1.5 eq.) and DIEA (3 eq.) were added. The reaction was allowed to slowly warm up to room temperature. After TLC analysis indicated disappearance of the Fmoc-alanine, the solvents were evaporated under reduced pressure and the remaining mixture was re-dissolved in EtOAc, washed with 5% HCl, 1 M $NaHCO_3$ and brine. The remaining organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Silica column chromatography 40% EtOAc in petroleum ether gave the desired Weinreb amide in a 97% yield. ESI-MS: [M+H]+: m/z = 355.17, obtained m/z = 354.90, $[M+Na]^+$ 377.15, obtained 376.90. ^1H NMR: to be measured on purer sample

N-(9-fluorenylmethoxycarbonyl)-L-alanal (10)

Weinreb amide **9** (1 eq.) was dissolved in dry THF (final concentration 0.15 M) under inert atmosphere at 0° C. $LiAlH_4$ (1.2 eq.) was slowly added and the mixture was stirred for 30 min. When TLC indicated complete conversion, the reaction was quenched by slow addition of 5% HCl until a pH of 3 was reached. THF was evaporated under reduced pressure and the aldehyde was extracted with EtOAc two times. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated, yielding the crude aldehyde in quantitative yield. ESI-MS: [M+H]+: m/z = 296.13, obtained m/z = 295.85; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.56 (s, 1H), 7.77 (d, J = 7.5 Hz, 3H), 7.60 (d, J = 7.4 Hz, 2H), 7.41 (t, J = 7.4 Hz, 3H), 7.32 (td, J = 7.3, 0.9 Hz, 3H), 5.43 (d, J = 4.9 Hz, 1H), 4.47 – 4.39 (m, 2H), 4.36 – 4.28 (m, 1H), 4.23 (t, J = 6.8 Hz, 1H), 1.38 (d, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 199.05, 155.86, 143.74, 141.35, 127.78, 127.11, 125.04, 120.04, 67.01, 55.93, 47.16, 14.87.

(2R/S, 3S)-3-*N*-(9-fluorenylmethoxycarbonyl)amino-2-hydroxy-3-methyl-propionitrile (11)

To a suspension of 12.7 mmol sodium cyanide (1.5 eq.) in 40 mL dry MeOH was added acetic acid (775 µL, 13.5 mmol, 1.6 eq.) and it was stirred for 5 min under Ar atmosphere at room temperature. It was then added to an ice-cooled solution of compound **10** (2.5 g, 1 eq.) in 40 mL dry DCM and the mixture was stirred for 1.5 h under inert atmosphere. When TLC indicated complete conversion, the reaction was quenched with 50 mL water, 40 mL brine and 10 mL saturated $NaHCO_3$ and stirred for 5 min. The organic layer was separated from the water layer, and the water layer was extracted with Et_2O (3x). The combined organic layers were washed with a 4:1 brine/saturated $NaHCO_3$ solution and additionally with 30 ml water, dried over $MgSO_4$ and filtered. Solvents in the filtrate were evaporated under reduced pressure. The crude material was immediately used for acidic hydrolysis in the next step. ESI-MS: [M+H]+: m/z = 323.14, obtained m/z = 322.85, $[M+Na]^+$ 345.12, obtained 344.85.

(2R/S, 3S)-3-*N*-(9-fluorenylmethoxycarbonyl)amino-2-hydroxy-3-methyl-propionic acid (12)

Compound **11** (9.56 mmol) was refluxed in 100 mL of a 1:1 solution of 1,4-dioxane and concentrated hydrochloric acid for two hours. When TLC indicated that the hydrolysis was finished, the reaction mixture was cooled down to room temperature and evaporated to dryness under reduced pressure. The residue was taken up in EtOAc (100 mL) and washed with 50 mL 5 % HCl. The organic phase was dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure. The crude material was purified by silica gel chromatography (40% EtOAc in petroleum ether + 1% AcOH), giving the title compound, as a diastereomeric mixture, in 47.5% yield. ESI-MS: [M+H]+: m/z = 342.14, obtained m/z = 341.90. HRMS: $[M-H]^-$ m/z = 340.1190, obtained m/z = 340.1169. ^1H NMR (CD_3OD; 300 MHz): 7.78 (d, J = 7.4 Hz, 2H), 7.68-7.60 (m, 2H), 7.38 (t, J = 7.3 Hz, 2H), 7.30 (t, J = 7.3 Hz, 2H), 4.39-4.05 (m, 5H), 1.31-1.06 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.5, 158.1, 145.3, 142.5, 128.7, 128.1, 126.3, 120.9, 73.6, 67.9, 50.8, 48.4, 14.7.

(3S)-3-*N*-(9-fluorenylmethoxycarbonyl)amino-2-keto-3-methyl-propionic acid (13)

Hydroxyacid **12** (1.0 eq.) was dissolved in DCM (final concentration 0.1 M). Dess-Martin periodinane (1.0 eq.) was added and the mixture was stirred at room temperature, until TLC indicated complete oxidation. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was purified by silica column chromatography (30-100% EtOAc in petroleum ether + 1% AcOH), giving the title compound in 43% yield, as a mixture of the keto-acid and its hydrate. ESI-MS: $[M+H]^+$: m/z = 339.12, obtained m/z = 338.90; $[M-H]^-$: m/z = 338.10, obtained m/z = 337.80; $[M-H+H_2O]^-$ m/z = 356.11, obtained m/z 355.80. HRMS: $[M-H]^-$ m/z = 338.1034, obtained m/z = 338.1026.

***N*-(9-fluorenylmethoxycarbonyl)-L-alanyl-L-alanine amide (15)**

Ketoacid **13** (1.0 eq.) was dissolved in DMF (final concentration 0.2 M), and HBTU (1.0 eq) and DIEA (3 eq.) were added. After several minutes of pre-activation, this mixture was added to L-alanine amide (1.5 eq.) and stirred under Argon. When TLC indicated disappearance of the ketoacid, the reaction was diluted with EtOAC, washed with sequentially with 5% HCl, 1M

NaHCO3 and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Crude material was analyzed by LC-MS (see Figure S2). ESI-MS: $[M\}H]^+$: m/z = 382.18, obtained m/z = 382.00.

General procedure for solid phase synthesis of peptide ketoamides

Peptidyl ketoamides were synthesized on Rink amide resin. All peptide couplings were executed using an automated peptide synthesizer (Syro II, Biotage). Fmoc groups were deprotected with 20% piperidine in DMF for 15 min. Couplings of amino acids were performed twice with 5 eq. of the respective Fmoc-amino acid, preactivated with HBTU (5 eq.) and DIEA (10 eq.) in DMF, except for building block **12**, which was coupled in a single step. After the final Fmoc-deprotection, the peptide was capped wit Ac_2O (5 eq.), Et_3N (5 eq) and pyridine (5 eq), in DMF. All next steps were performed by manual solid phase synthesis in a 1 mL cartridge. Naturally, the treatment with Ac_2O resulted in acetylation of the secondary alcohol of building block **12**. The acetyl was removed by treatment of the resin with 1 M KOH in MeOH for 1 hour at room temperature. After thorough washing with methanol, DCM and DMF, de-acetylation was checked by LC-MS by taking an aliquot of resin and cleaving the intermediate from the resin. Next, the secondary alcohol was oxidized with IBX (3 eq.) in the presence of H_2O (3 eq.) in DMF:DMSO (1:1). The mixture was left overnight at room temperature. Finally, the products were cleaved from the resin by TFA/H_2O/TIS 95/2.5/2.5 (v/v/v), evaporated under reduced pressure and purified by reversed phase HPLC. Fractions with product were pooled, frozen in liquid nitrogen and lyophilized to dryness.

Ac-Val-Arg-His-Ala(α-ketoamide)-Ala-NH_2 (18)

The title compound was synthesized according to the general procedure and obtained as a white, fluffy solid in 8.7% yield, based on the original Rink resin loading. ESI-MS: [M+H]+: m/z = 622.34, obtained m/z = 622.15. HRMS: [M+H]+: m/z = 622.3420, obtained m/z = 622.3434; $[M+H_2O+H]^+$ = 640.3525, obtained m/z = 640.3541

Ac-Val-Arg-His-Ala(α-ketoamide)-Ala-Phe-NH_2 (19)

The title compound was synthesized according to the general procedure and obtained as a white, fluffy solid in 12.7% yield, based on the original Rink resin loading. ESI-MS: $[M+H]^+$: m/z = 769.41 , obtained m/z = 769.25, $[M+H_2O+2H]^{2+}$: m/z 394.21, obtained m/z = 394.15. HRMS: [M+H]+: m/z = 769.4104, obtained m/z = 769.4105; $[M+H_2O+H]^+$ = 787.4209, obtained m/z = 787.4217

Ac-Ile-Ala-Thr-Ala(α-ketoamide)-Ala-Phe-NH_2 (20)

The title compound was synthesized according to the general procedure and obtained as a white, fluffy solid in 1.9% yield, based on the original Rink resin loading. ESI-MS: [M+H]+: m/z = 662.35, obtained m/z = 662.15 HRMS: [M+H]+: m/z = 662.3508, obtained m/z = 662.3517

Inhibitor testing by competitive activity-based protein profiling

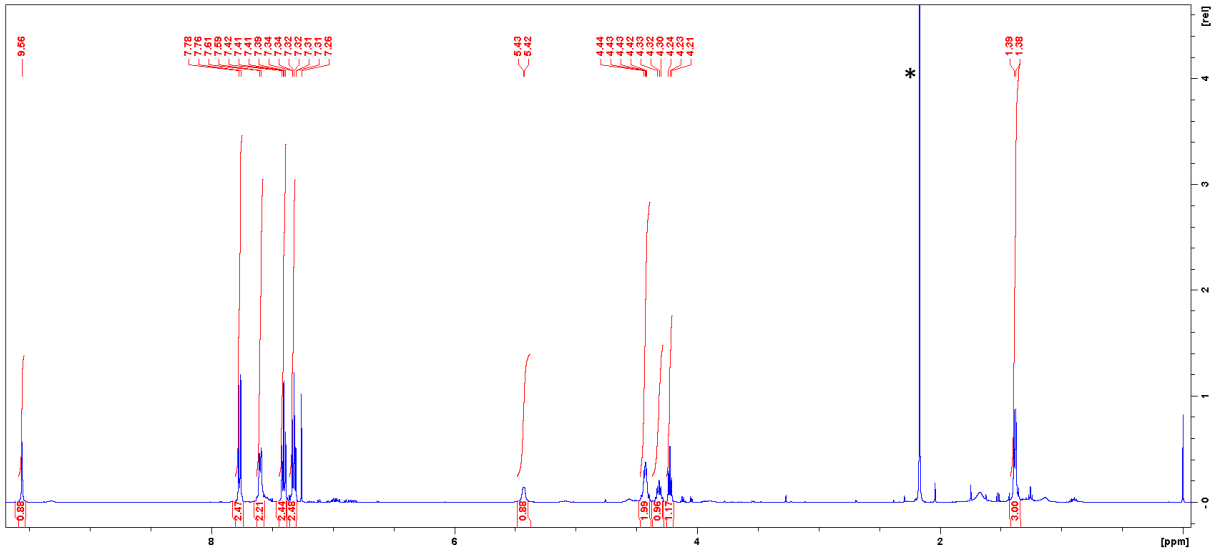
E. coli rhomboid protease GlpG was expressed and purified as described before.[1] 60 ng purified GlpG in 30 µL reaction buffer (20 mM HEPES, pH 7.5, 10% (v/v) glycerol and 0.05% (w/v) DDM) was incubated for 30 min at room temperature with compound (50 µM **5**-**7**, **18**-**20**, 100 µM DCI or an equal volume of DMSO (1% v/v), respectively. Next, FP-Rh was added to a final concentration of 1 µM and incubated for an additional hour at room temperature in the dark. The reaction was stopped with 1/3rd of a volume of 4× Laemmli buffer. Half of the mixture was resolved by 15% SDS-PAGE. The resulting gels were scanned by using a Typhoon FLA 9500.

Supplemental references

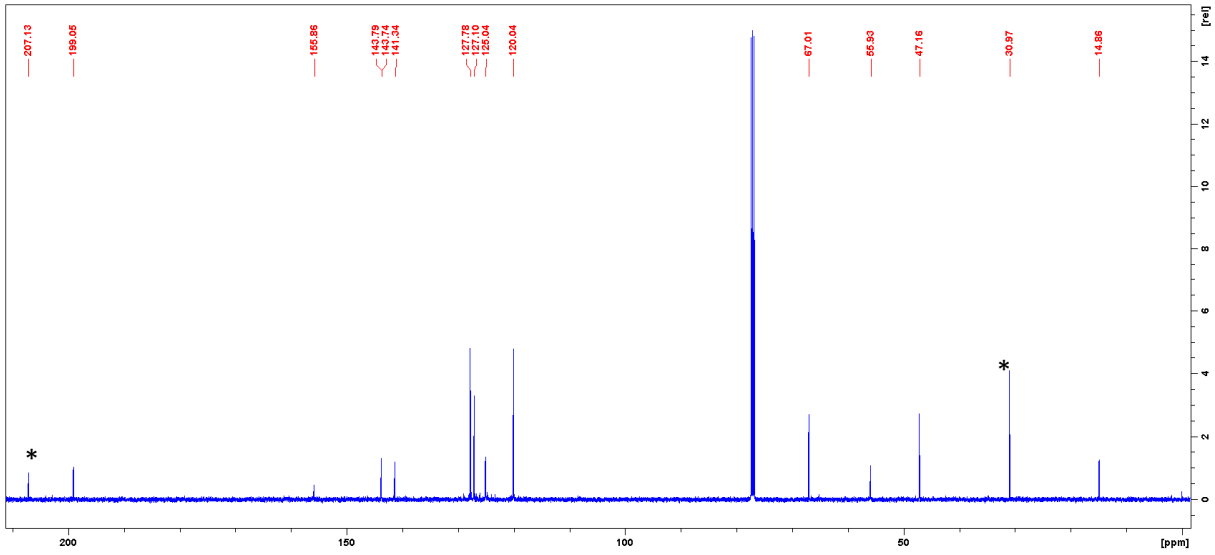
1. Vosyka, O.; Vinothkumar, K. R.; Küttler, E. V., Brouwer, A. J.; Liskamp, R. J. M.; Verhelst, S. H. L. (2013) Activity-based probes for rhomboid proteases discovered in a mass spectrometry-based assay. *Proc. Natl. Acad. Sci. USA*, 110: 2472-2477.

Copies of spectra

^{1}H NMR spectrum of compound **10**

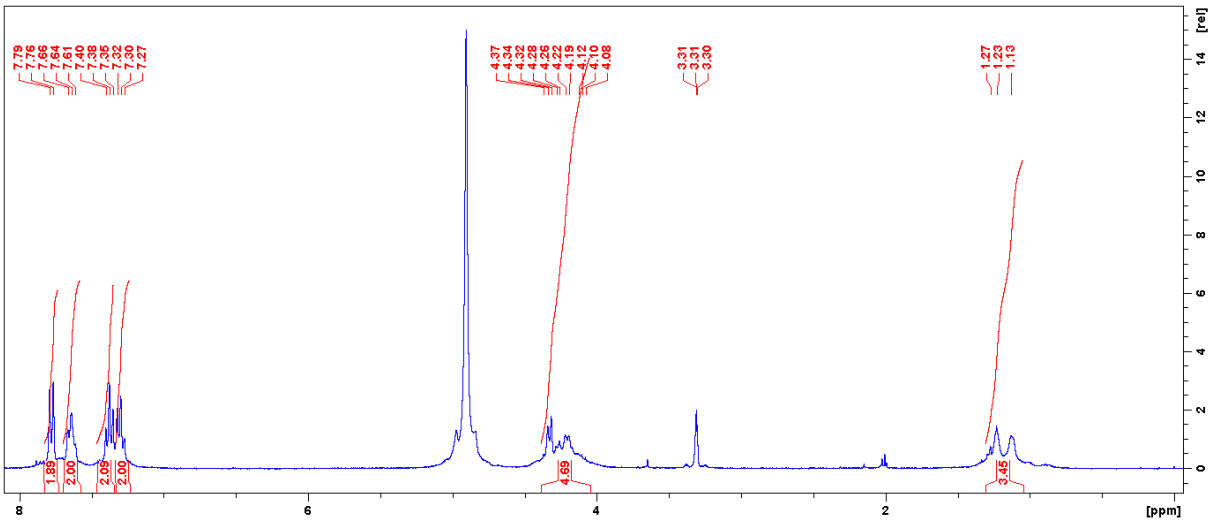


^{13}C NMR spectrum of compound **10**

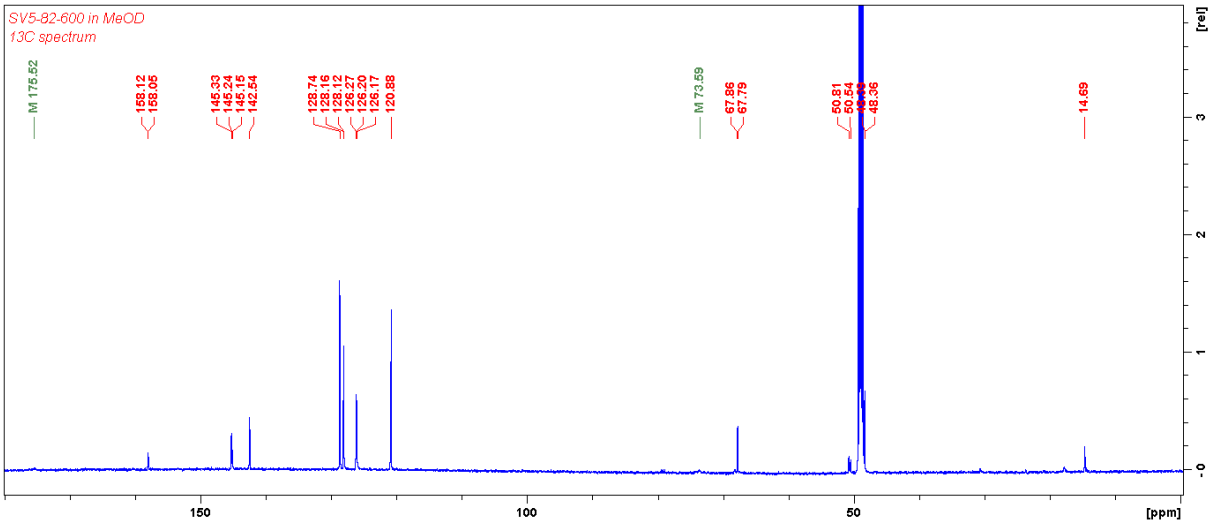


* indicates acetone

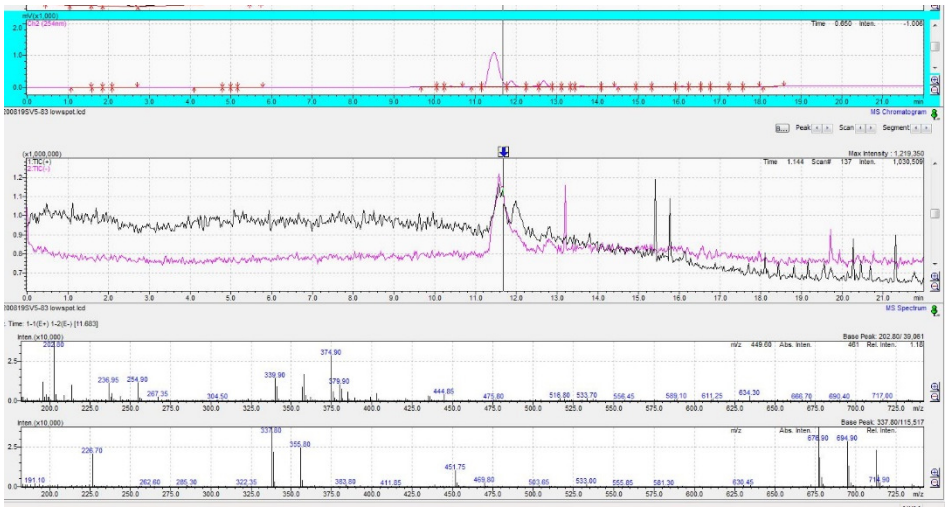
^{1}H NMR spectrum of compound **12**



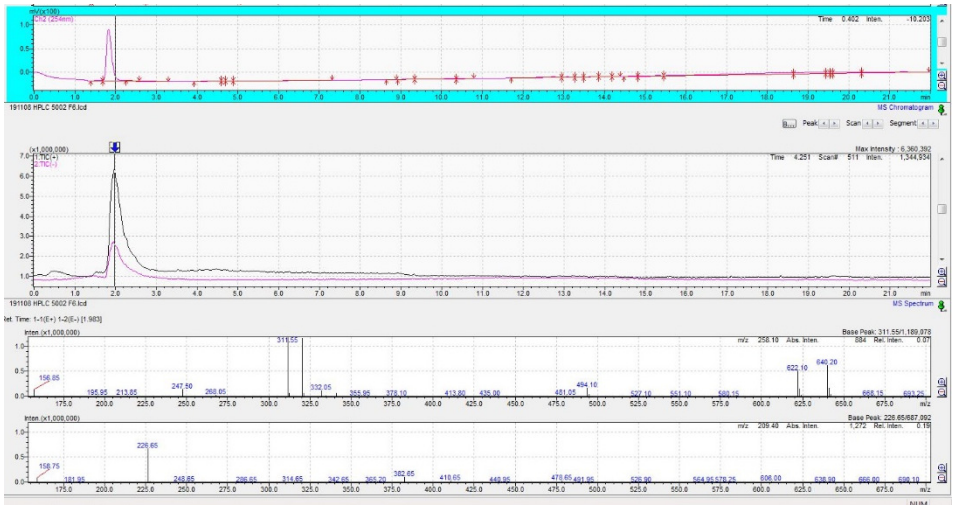
^{13}C NMR spectrum of compound **12**



LC chromatogram and MS spectrum of compound **13**



LC chromatogram and MS spectrum of compound **18**



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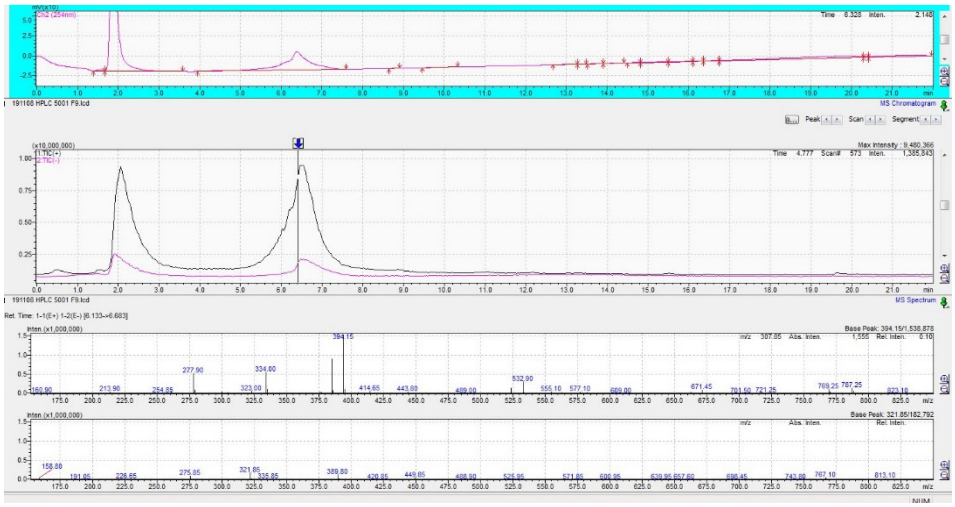

LC chromatogram and MS spectrum of compound **20**