Electronic Supplementary Material (ESI) for ChemComm.

Peptide-triggered IL-12 and IFN-γ mediated immune response in $CD4^+$ T-cells against *Leishmania donovani* infection

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Abstract:*Leishmania donovani* are intracellular, human blood parasites that cause visceral leishmaniasis or kala-azar.Cell-penetrating peptides (CPPs) modulate intracellular processes, cargo delivery whereas host defense peptides (HDPs) promote proliferation of both naïve and antigen activated $CD4^+$ T-cells.We reportnewly designed tripeptides that were able to trigger proinflammatory cytokines (IL-12 and IFN-γ) secretion by $CD4^+CD44^+$T-cells in response to*Leishmania donovani* infection. These peptides accelerating specific T_H1 responses can combat obstacles of cytotoxicity and drug resistance.We demonstrate that small peptide-mediated cytokines secretion could be an effective immunotherapeutic approach for immunity restoration against visceral leishmaniasis.

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Experimental Procedures

General

Dichloromethane (DCM), N, N'-dimethylformamide (DMF), acetonitrile (CH_3CN), triethylamine (TEA), and methanol (MeOH) were distilled following standard procedures prior to use. Trifluoroacetic acid was obtained from Spectrochem, L-amino acids and 2-Chlorotrityl chloride resin were obtained from Sigma Aldrich and used without further purification. D-amino acids were purchased from TCI Chemicals Pvt. Ltd. ^{1}H and ^{13}C NMR spectra were recorded on JEOL-JNM ECS 400 model operating at 400 and 100 MHz, respectively. HRMS spectra were recorded at IIT Kanpur, India, on Waters, Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6-3.2 Kv.

Peptide Synthesis: Peptides **1**, **2, 3, 4, 5, and 6** were synthesized by standard solid-phase peptide synthesis method and analytically characterized for purity as per our previous protocol.[1,2] All fine chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and TCI Chemicals Pvt. Ltd.

Synthesis of D-Cysteinyl-D-alanyl-glycine (1): 2-Chlorotrityl chloride resin (152 mg, 0.2 mmol, having substitution 1.32 mmol/gm) was swollen for 30 minutes in dry DCM (3 mL). Resin was then washed with dry DCM (3x3 mL). Solution of Fmoc–Gly-OH (267.5 mg, 0.9 mmol) and DIPEA (209 µL, 1.2 mmol) in 4 mL of dry DCM was added to the resin and reaction mixture was stirred for 3 h by purging nitrogen gas. To endcap the reactive group on resin HPLC grade methanol (1 mL) was added to the solution and stirred for 15 minutes. After that, the resin was washed with DMF (3x3 mL) and DCM (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. The resin was then washed with DMF (3x3 mL). To this resin a solution of Fmoc-Ala-OH (280.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Coupling was monitored by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was washed with DMF (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen. The resin was washed with DMF (3x3 mL). After proper washing of resin a solution of Fmoc-Cys(Trt)-OH (527.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for additional 4 h under nitrogen. Sample of resin was tested for monitoring the coupling by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. The resin after proper washing was treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. After completion of reaction, the resin was then washed with DMF (3x3 mL) and DCM (3x3 mL). The resin obtained was then dried under vacuum. The resin after proper drying was then treated with 10 mL mixture of TFA/TIPS/EDT/Water/DCM (30/2.5/2.5/2.5/62.5%) and the reaction mixture was stirred for 1 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. The crude peptide was further purified by reversed phase HPLC (RP-HPLC) using solvent system 0.1 % TFA in water/acetonitrile with C-18 column and then the sample was lyophilized to give the desired tripeptide as a white powder. Yield (37.4 mg, 0.15 mmol, 75%); white colour solid. ESI-HRMS: $[M+H]^+$ calculated = 250.0862, found = 250.0861. ^{1}H NMR (400 MHz, D_2O) δ 4.31 (dd, *J* = 14.4, 7.2 Hz, 1H), 4.13 (t, *J* = 5.4 Hz, 1H), 3.89 (q, *J* = 18.0 Hz, 2H), 2.99 (qd, *J* = 15.1, 5.6 Hz, 2H), 1.32 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-D6) δ 172.77 (s), 171.54 (s), 166.92 (s), 54.26 (s), 48.92 (s), 41.12 (s), 25.84 (s), 18.52 (s).

Synthesis of L-Cysteinyl-L-alanyl-glycine (2): The above protocol was followed for synthesis using D-amino acids.ESI-HRMS: $[M-H]^+$ calculated = 248.07, found = 248.0702. ^{1}H NMR (400 MHz, D_2O) δ 4.66 (s, 7H), 4.31 (q, *J* = 7.2 Hz, 1H), 4.13 (t, *J* = 5.5 Hz, 1H), 3.89 – 3.73 (m, 2H), 2.98 (qd, *J* = 15.0, 5.5 Hz, 2H), 1.31 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 174.68 (s), 174.09 (s), 167.74 (s), 54.06 (s), 49.82 (s), 41.77 (s), 25.04 (s), 16.46 (s).

Synthesis of L-Cysteinyl-L-alanyl-glycinyl-L-cysteine (3): 2-Chlorotrityl chloride resin (152 mg, 0.2 mmol, having substitution 1.32 mmol/gm) was swollen for 30 minutes in dry DCM (3 mL). Resin was then washed with dry DCM (3x3 mL). Solution of Fmoc–Cys (Trt)-OH (527.1 mg, 0.9 mmol) and DIPEA (209 µL, 1.2 mmol) in 4 mL of dry DCM was added to the resin and reaction mixture was stirred for 3 h by purging nitrogen gas. To endcap the reactive group on resin HPLC grade methanol (1 mL) was added to the solution and stirred for 15 minutes. After that, the resin was washed with DMF (3x3 mL) and DCM (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. The resin was then washed with DMF (3x3 mL). To this resin a solution of Fmoc-Gly-OH (267.5 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Coupling was monitored by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was washed with DMF (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen. The resin was washed with DMF (3x3 mL). To this resin a solution of Fmoc-Ala-OH (280.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Coupling was monitored by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was washed with DMF (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen. The resin was washed with DMF (3x3 mL). After proper washing of resin, a solution of Fmoc-Cys (Trt)-OH (527.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for additional 4 h under nitrogen. Sample of resin was tested for monitoring the coupling by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. The resin after proper washing was treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. After completion of reaction, the resin was then washed with DMF (3x3 mL) and DCM (3x3 mL). The resin obtained was then dried under vacuum. The resin after proper drying was then treated with 10 mL mixture of TFA/TIPS/EDT/Water/DCM (30/2.5/2.5/2.5/62.5%) and the reaction mixture was stirred for 1 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. The crude peptide was further purified by reversed phase HPLC (RP-HPLC) using solvent system 0.1 % TFA in water/acetonitrile with C-18 column and then the sample was lyophilized to give the desired tripeptide as a white powder. Yield (40 mg, 0.15 mmol, 74%); white colour solid. ESI-HRMS: $[M+DMSO+H]^+$calculated = 431.108, found = 431.195. ^{1}H NMR (400 MHz, D_2O) δ 4.59 – 4.50 (m, 1H), 4.29 (q, *J* = 7.2 Hz, 1H), 4.13 (t, *J* = 5.0 Hz, 1H), 3.89 (s, 2H), 2.99 (qd, *J* = 14.9, 5.5 Hz, 2H), 2.91 – 2.87 (m, 2H), 1.32 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 175.11 (s), 173.28 (s), 171.10 (s), 167.84 (s), 54.36 (d, *J* = 65.9 Hz), 49.99 (s), 42.38 (s), 36.93 (s), 25.16 (d, *J* = 16.8 Hz), 16.39 (s).

Synthesis of L-Cysteinyl-L-valinyl-glycine (4): 2-Chlorotrityl chloride resin (152 mg, 0.2 mmol, having substitution 1.32 mmol/gm) was swollen for 30 minutes in dry DCM (3 mL). Resin was then washed with dry DCM (3x3 mL). Solution of Fmoc–Gly-OH (267.5 mg, 0.9 mmol) and DIPEA (209 µL, 1.2 mmol) in 4 mL of dry DCM was added to the resin and reaction mixture was stirred for 3 h by purging nitrogen gas. To endcap the reactive group on resin HPLC grade methanol (1 mL) was added to the solution and stirred for 15 minutes. After that, the resin was washed with DMF (3x3 mL) and DCM (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. The resin was then washed with DMF (3x3 mL). To this resin a solution of Fmoc-Ile-OH (280.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Coupling was monitored by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was washed with DMF (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen. The resin was washed with DMF (3x3 mL). After proper washing of resin, a solution of Fmoc-Cys (Trt)-OH (527.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for additional 4 h under nitrogen. Sample of resin was tested for monitoring the coupling by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. The resin after proper washing was treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. After completion of reaction, the resin was then washed with DMF (3x3 mL) and DCM (3x3 mL). The resin obtained was then dried under vacuum. The resin after proper drying was then treated with 10 mL mixture of TFA/TIPS/EDT/Water/DCM (30/2.5/2.5/2.5/62.5%) and the reaction mixture was stirred for 1 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. The crude peptide was further purified by reversed phase HPLC (RP-HPLC) using solvent system 0.1 % TFA in water/acetonitrile with C-18 column and then the sample was lyophilized to give the desired tripeptide as a white powder. Yield (38 mg, 78%); white colour solid. ESI- HRMS: $[M+Isoprop+H]^+$ calculated = 338.34, found = 338.341. ^{1}H NMR (400 MHz, METHANOL-D3) δ 4.25 (d, *J* = 7.1 Hz, 1H), 4.16 (t, *J* = 5.6 Hz, 1H), 3.99 – 3.81 (m, 2H), 3.05 – 2.95 (m, 2H), 2.09 (dq, *J* = 13.7, 6.8 Hz, 1H), 0.98 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, METHANOL-D3) δ 172.35 (s), 171.33 (s), 167.29 (s), 59.32 (s), 54.42 (s), 40.45 (s), 30.46 (s), 25.27 (s), 18.31 (s), 17.31 (s).

Synthesis of L-Cysteinyl-L-leucinyl-glycine (5): 2-Chlorotrityl chloride resin (152 mg, 0.2 mmol, having substitution 1.32 mmol/gm) was swollen for 30 minutes in dry DCM (3 mL). Resin was then washed with dry DCM (3x3 mL). Solution of Fmoc–Gly-OH (267.5 mg, 0.9 mmol) and DIPEA (209 µL, 1.2 mmol) in 4 mL of dry DCM was added to the resin and reaction mixture was stirred for 3 h by purging nitrogen gas. To endcap the reactive group on resin HPLC grade methanol (1 mL) was added to the solution and stirred for 15 minutes. After that, the resin was washed with DMF (3x3

mL) and DCM (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. The resin was then washed with DMF (3x3 mL). To this resin a solution of Fmoc-Leu-OH (280.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Coupling was monitored by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was washed with DMF (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen. The resin was washed with DMF (3x3 mL). After proper washing of resin, a solution of Fmoc-Cys (Trt)-OH (527.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for additional 4 h under nitrogen. Sample of resin was tested for monitoring the coupling by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. The resin after proper washing was treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. After completion of reaction, the resin was then washed with DMF (3x3 mL) and DCM (3x3 mL). The resin obtained was then dried under vacuum. The resin after proper drying was then treated with 10 mL mixture of TFA/TIPS/EDT/Water/DCM (30/2.5/2.5/2.5/62.5%) and the reaction mixture was stirred for 1 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. The crude peptide was further purified by reversed phase HPLC (RP-HPLC) using solvent system 0.1 % TFA in water/acetonitrile with C-18 column and then the sample was lyophilized to give the desired tripeptide as a white powder. Yield (40 mg, 80%); white colour solid. ESI- HRMS: $[M+H]^+$calculated = 292.133, found = 292.1318. ^{1}H NMR (400 MHz, METHANOL-D3) δ 4.46 (t, *J* = 7.5 Hz, 1H), 4.10 (t, *J* = 5.5 Hz, 1H), 3.98 – 3.79 (m, 2H), 3.10 – 2.96 (m, 2H), 1.70 (td, *J* = 13.2, 6.5 Hz, 1H), 1.61 (t, *J* = 7.3 Hz, 2H), 0.93 (dd, *J* = 10.5, 6.4 Hz, 6H). ^{13}C NMR (101 MHz, METHANOL-D3) δ 173.48 (s), 171.36 (s), 167.16 (s), 54.39 (s), 52.12 (s), 40.45 (s), 25.18 (s), 24.45 (s), 22.05 (s), 20.56 (s).

Synthesis of L-Cysteinyl-L-isoleucinyl-glycine (6): 2-Chlorotrityl chloride resin (152 mg, 0.2 mmol, having substitution 1.32 mmol/gm) was swollen for 30 minutes in dry DCM (3 mL). Resin was then washed with dry DCM (3x3 mL). Solution of Fmoc–Gly-OH (267.5 mg, 0.9 mmol) and DIPEA (209 µL, 1.2 mmol) in 4 mL of dry DCM was added to the resin and reaction mixture was stirred for 3 h by purging nitrogen gas. To endcap the reactive group on resin HPLC grade methanol (1 mL) was added to the solution and stirred for 15 minutes. After that, the resin was washed with DMF (3x3 mL) and DCM (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. The resin was then washed with DMF (3x3 mL). To this resin a solution of Fmoc-Ile-OH (280.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Coupling was monitored by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was washed with DMF (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen. The resin was washed with DMF (3x3 mL). After proper washing of resin, a solution of Fmoc-Cys (Trt)-OH (527.1 mg, 0.9 mmol) and HOBt (121 mg, 0.9 mmol) in 4 mL of DMF was added followed by addition of DIC (141 µL, 0.9 mmol) and the reaction mixture was stirred by purging nitrogen gas for additional 4 h under nitrogen. Sample of resin was tested for monitoring the coupling by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. The resin after proper washing was treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. After completion of reaction, the resin was then washed with DMF (3x3 mL) and DCM (3x3 mL). The resin obtained was then dried under vacuum. The resin after proper drying was then treated with 10 mL mixture of TFA/TIPS/EDT/Water/DCM (30/2.5/2.5/2.5/62.5%) and the reaction mixture was stirred for 1 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. The crude peptide was further purified by reversed phase HPLC (RP-HPLC) using solvent system 0.1 % TFA in water/acetonitrile with C-18 column and then the sample was lyophilized to give the desired tripeptide as a white powder. Yield (38 mg, 78%); white colour solid. ESI- HRMS: $[M+Na]^+$calculated = 314.115, found = 314.1167.^{1}H NMR (400 MHz, METHANOL-D3) δ 4.28 (d, *J* = 7.5 Hz, 1H), 4.10 (t, *J* = 5.7 Hz, 1H), 3.89 (dd, *J* = 45.1, 17.7 Hz, 2H), 3.00 (d, *J* = 5.1 Hz, 2H), 1.91 – 1.79 (m, 1H), 1.58 (dqd, *J* = 15.0, 7.5, 3.4 Hz, 1H), 1.26 – 1.12 (m, 1H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, METHANOL-D3) δ 172.33 (s), 171.19 (s), 167.10 (s), 58.29 (s), 54.42 (s), 40.38 (s), 36.69 (s), 25.24 (s), 24.52 (s), 14.45 (s), 10.09 (s).

High-performance liquid chromatography (HPLC)

HPLC analyses were performed with a HPLC system (Agilent technologies 1260 infinity) equipped with a quaternary pump (G1311B), auto liquid sampler (G1329B), Diode array detector (G1315D) and analytical scale fraction collector (G1364C). Instrumental control, data acquisition, and processing were performed using ChemStation software (Agilent Technologies, Wokingham, UK). A ZORBAX Eclipse plus C18 (250 x 4.6 mm) column with 5 µm particle size at room temperature was used. Acetonitrile/water (10:90) were used as mobile phase and the flow rate was 1 ml/min. Injection volume was 20 µL and effluent was measured at 220 nm.

Cell culture reagents

Animals and parasites

Five to six-week-old female BALB/c mice from Central Drug Research Institute, Lucknow, India was used in the experiments. All procedures, related to animals and parasites used in this study, were reviewed and approved (No. F.Sc./88/IAEC/2016-17/174) by the Ethics Committee of the Institute of Science, Banaras Hindu University, Varanasi as per CPCSEA guidelines of the Government of India, India. A cloned line of *L. donovani* was used, and parasites virulence were maintained in hamsters. *Leishmania donovani* promastigotes were cultured at 26°C in M199 media (pH7.2-7.5) supplemented with antibiotics Penicillin 100U/ml, streptomycin 100mg/ml, gentamycin 20mg/ml, 0.1% hemin, 10 mM adenine and 10% FBS. Density gradient centrifugation was done to purify the metacyclic forms of *L. donovani* for animal infections.[3] All the cultured parasites were

centrifuged and washed 3 times in PBS before infecting animals. Animals were infected with $3x10^6$ parasites *via* tail vein whenever needed to obtain splenocytes as per experimental requirements.

T-cell proliferation assay

Pan T cells were isolated from spleen of control and infected (at day-14) animals, processed by disruption of the tissue with a syringe plunger into a single cell suspension in incomplete media (RPMI/DMEM) and filtered with 70µM strainer. Further cells were incubated with RBC lysis buffer for 3 minutes at 37^0C to remove RBCs. Cells were washed three times at 500g for 5-10 min with 10mM PBS (pH-7.2). The splenocytes were cultured in RPMI media that was supplemented with 10% FBS (Gibco, USA) and antibiotics (Sigma, USA) in a CO_2 incubator at 37^0C for the desired time points in presence or absence of the desired concentration of peptides, LPS and SLA. Cell proliferation were measured either by MTT assay or CFSE staining (quantified on Attune NxT Flow cytometer, Thermo Fisher, USA).

MTT assay was done as per standard protocol described elsewhere.[4] Briefly, pan T cells were seeded in 96 well plate in the presence of SLA and synthesised peptides then incubated for 5 days at 37^0C in 5% CO_2 atmosphere. After incubation 30µl (stock 5000µg/ml) MTT was added to each well and further incubated for 4 hours at 37^0C. Next, 100 µl DMSO was added to each well to dissolve formazan produced by viable cells. Accordingly, absorbance at 540 nm was measured by Elisa plate reader which represented the cells viability.

For CFSE staining, cells after washing were stained with 5 µM carboxyfluorescein diacetate succinimidyl ester (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen) followed by incubation for 30 min at room temperature in dark. The reaction was stopped by adding complete RPMI media (containing 10% FBS) and washed three times at 500g for 5-10 min to remove all traces of unbound dye. Cells were seeded in 96-well plates and stimulated with soluble leishmanial antigens (SLA) and different synthesized peptides **1, 2, 3, 4, 5** and **6**. After 5 days, CFSE stained cells were harvested and analysed with Attune NxT Flow Cytometer (Thermo Fisher, USA).

Measurements of pro-inflammatory cytokines by flow cytometry

We also checked if these peptides are capable to induce the production of inflammatory cytokines (IL-12 and IFN-γ in this case) by antigen experienced $CD4^+$ T cells i.e., $CD4^+CD44^+$ T cells either by intracellular cytokine staining, which was quantified by flow cytometer or by qPCR. For intracellular staining, T cells from infected and non-infected animals were first stained with surface molecules i.e. anti-CD3-eFluor 506, anti-CD4-Superbright 702 and CD44-Superbright 436, after surface staining cells were fixed, permeabilized and stained with anti-IL12-eFluor 660 and IFNγ-APC-eFluor780 antibodies. Finally, cells were acquired on flow cytometry and cytokines $CD4^+CD44^{+high}$ and $CD4^+CD44^{+low}$ were measured.

Measurement of cytokines in splenocytes treated with various peptides by qPCR

The expression levels of selected pro-inflammatory (IL-12, IFN- γ) cytokines, in antigen (Ag) experienced and non-antigen (nAg) experienced splenocyte cells were measured by quantitative PCR using gene specific primers (Table 1).Briefly, after treatment, cells were washed with PBS and total RNA was extracted using TRI® (Sigma-Aldrich, USA) reagent as per manufacturer's protocol. For expression analysis of genes in different experimental conditions, the cDNA was synthesized from 1µg of total RNA of each experimental replicate using cDNA synthesis kit (Applied Biosystems, USA) as per manufacturer's protocol. The mRNAs were amplified on Applied Biosystems fast 7500 Detection system with SYBR green qPCR master mix as per manufacturer's instructions (Applied Biosystems, USA). All reactions were performed in triplicate, and negative controls (no template cDNA) were included in each experiment. GAPDH was taken as internal control and all the data sets were normalized to the level of GAPDH. Fold change in gene expression was calculated by Δ^2CT method and results were reported as arbitrary unit or fold changes.

Results and Discussion

HPLC spectra

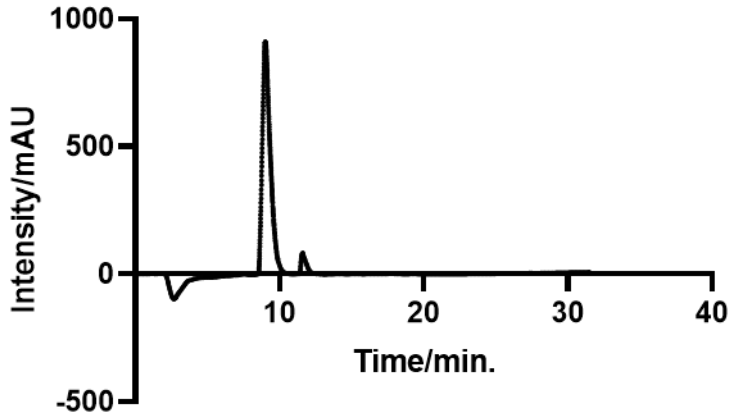


Fig. S1 HPLC spectrum of **1.**

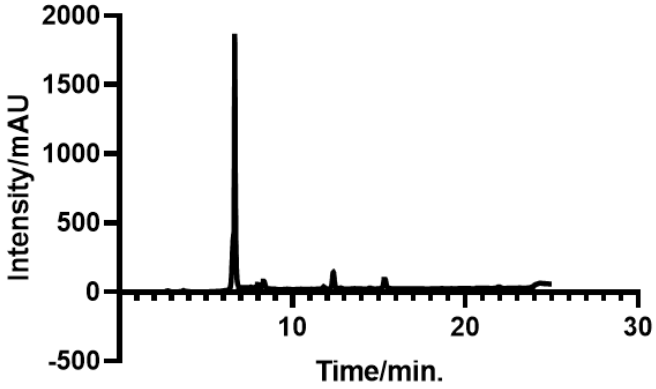


Fig. S2 HPLC spectrum of 2.

MTT assay

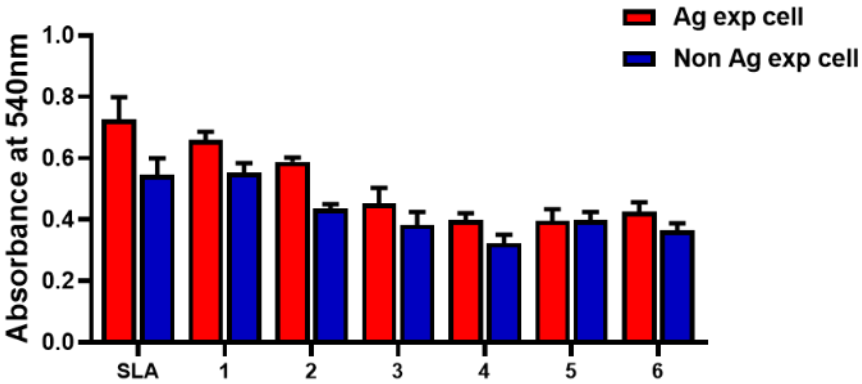


Fig. S3 Cell proliferation determined by MTT assay after 5 days of incubation on splenocyte and presented as absorbance at 540nm at 50 nM concentration.

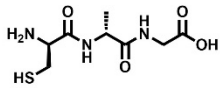
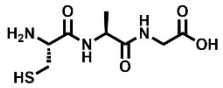
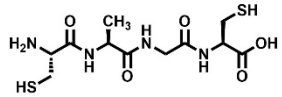
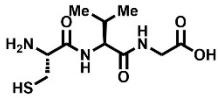
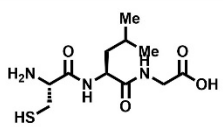
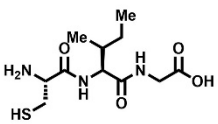
Peptide structures	Notations
	1
	2
	3
	4
	5
	6

Table S1: Structures of analogs

^{1}H NMR, ^{13}C NMR and ESI-MS Spectra

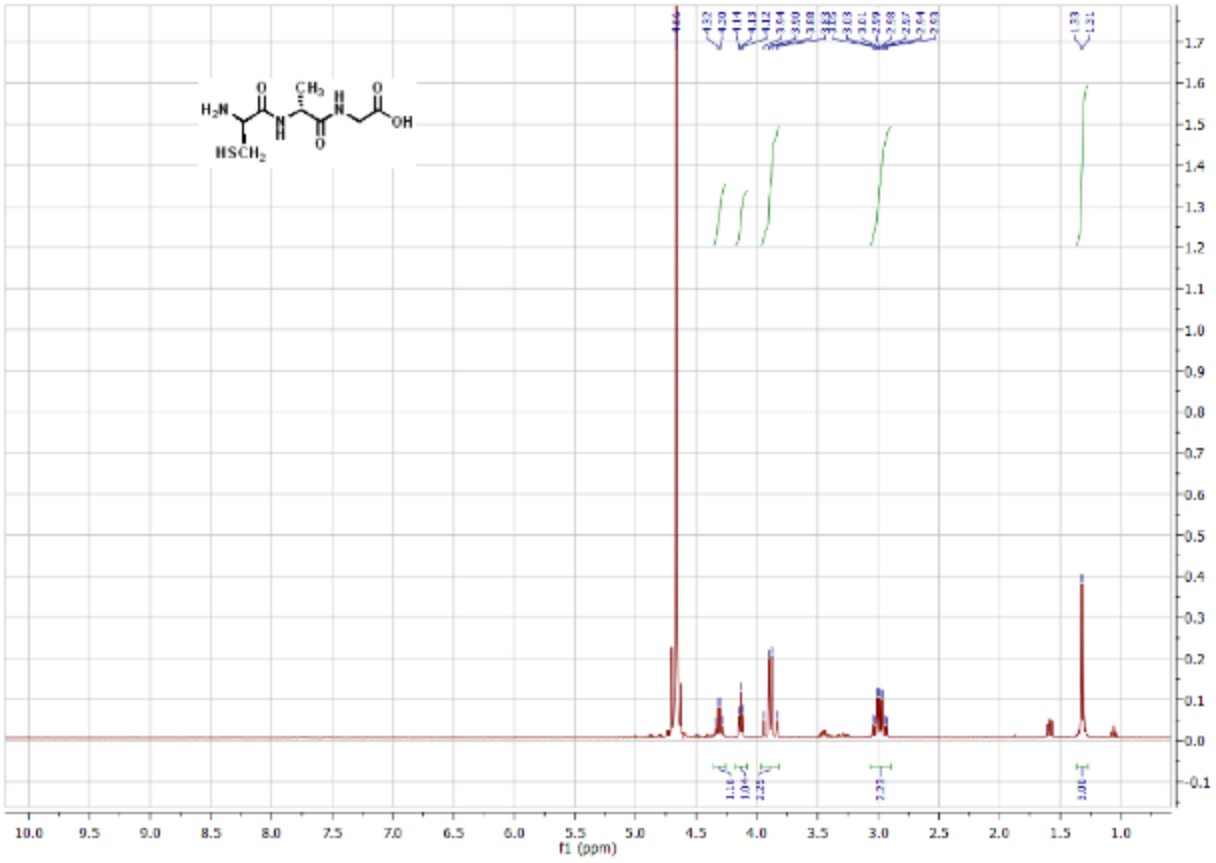


Fig. S6^{1}H spectrum of 1.

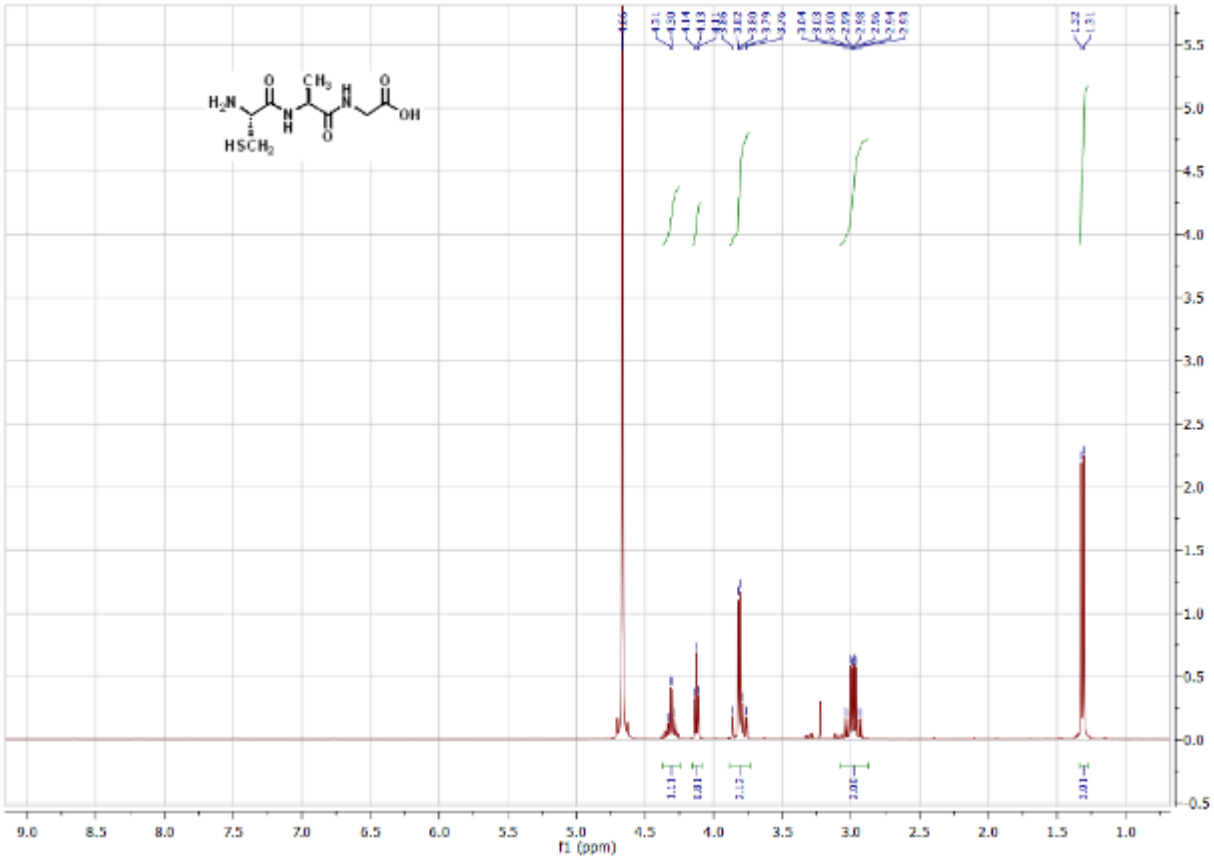


Fig. S7^{1}H spectrum of **2**.

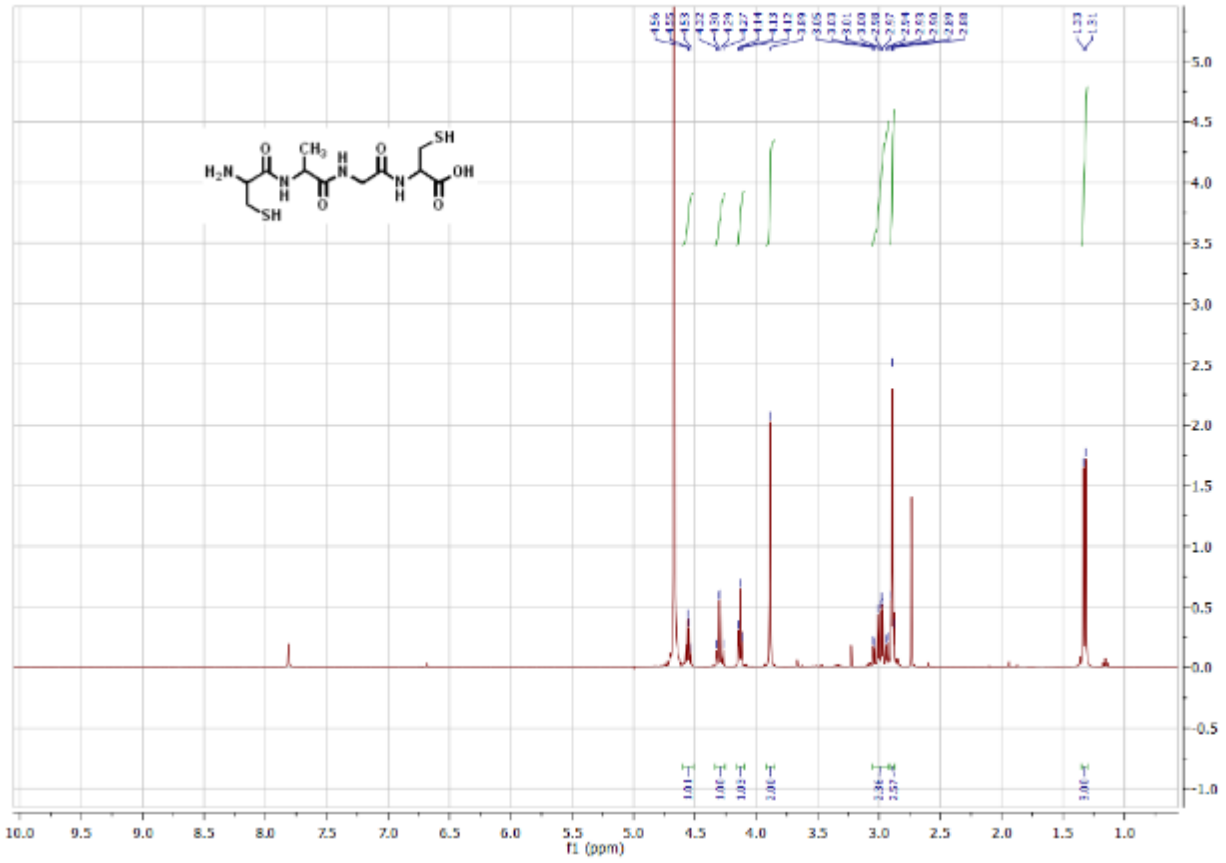


Fig. S8 ^{1}H spectrum of**3.**

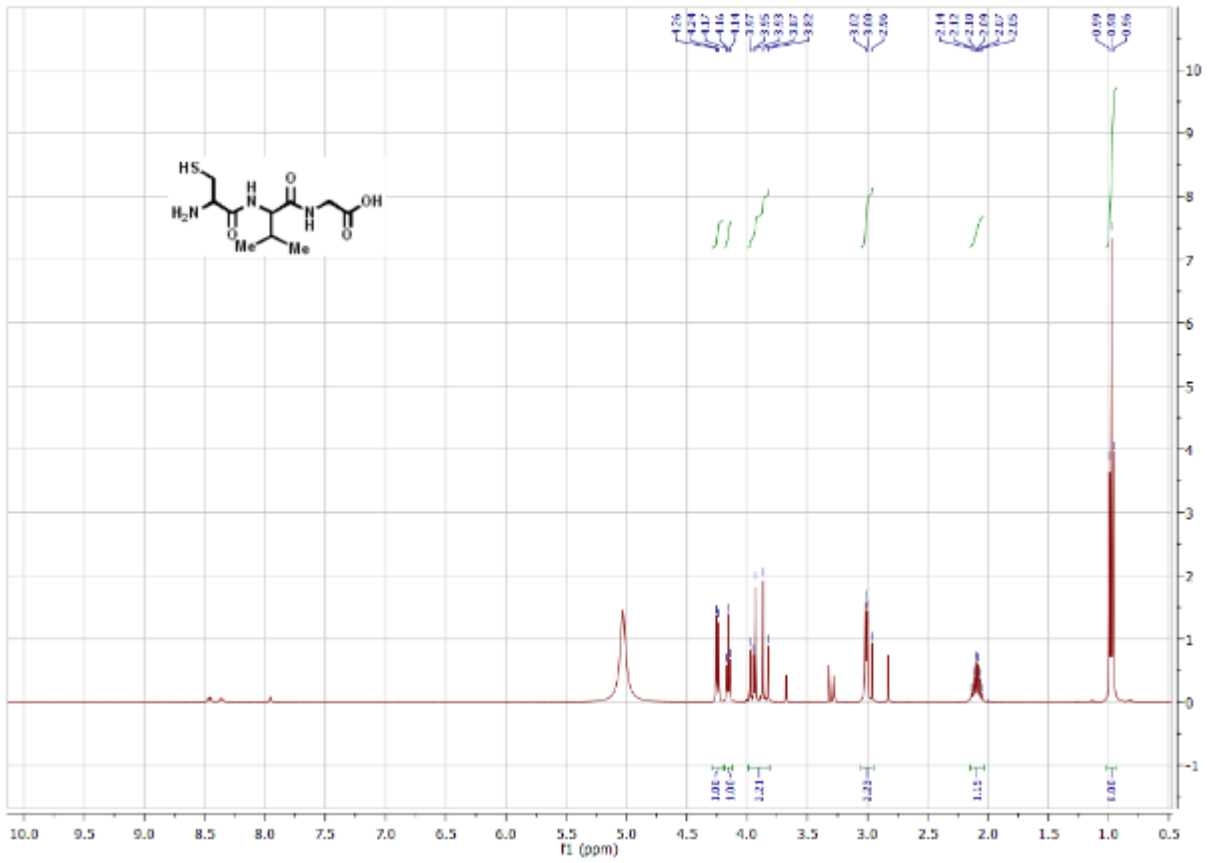


Fig. S9 ^{1}H spectrum of **4.**

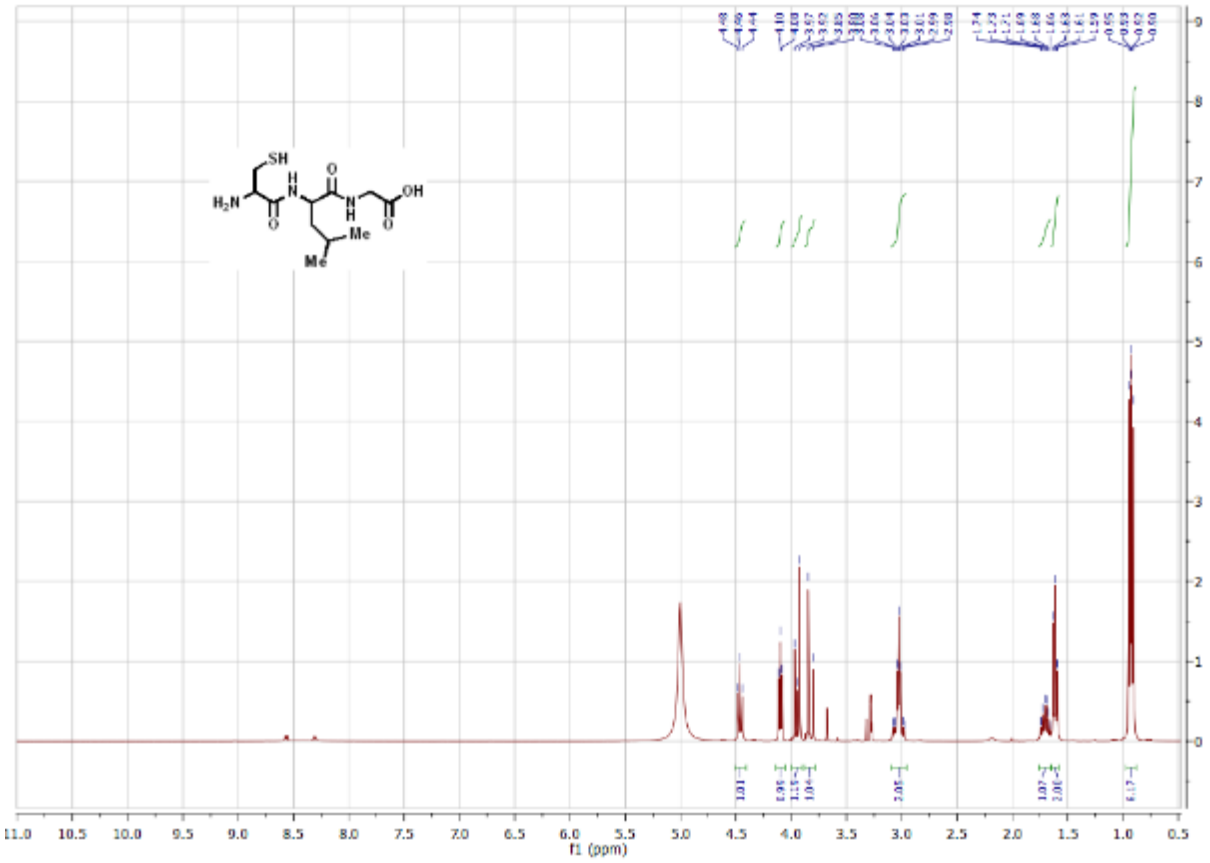


Fig. S10 ^{1}H spectrum of **5.**

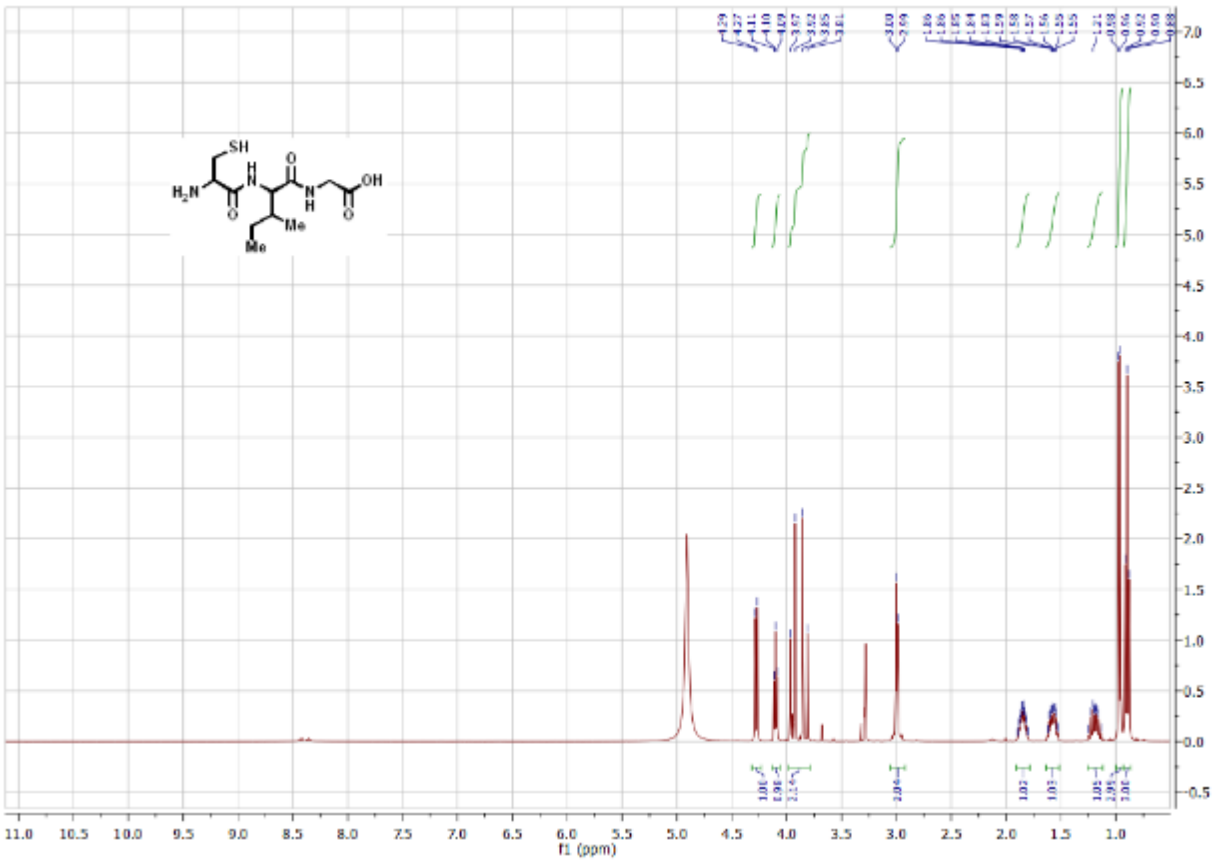


Fig. S11 ^{1}H spectrum of **6.**

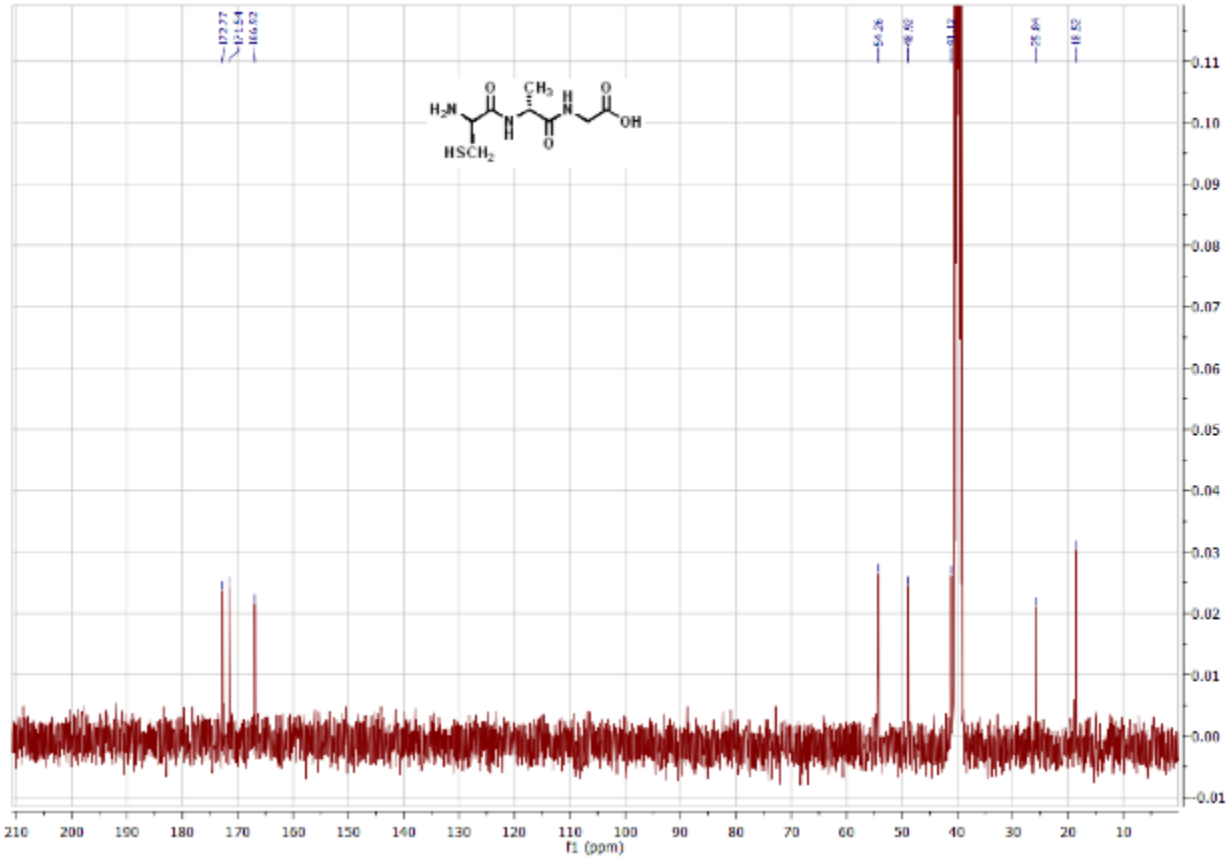


Fig. S12 ^{13}C NMR spectrum of **1.**

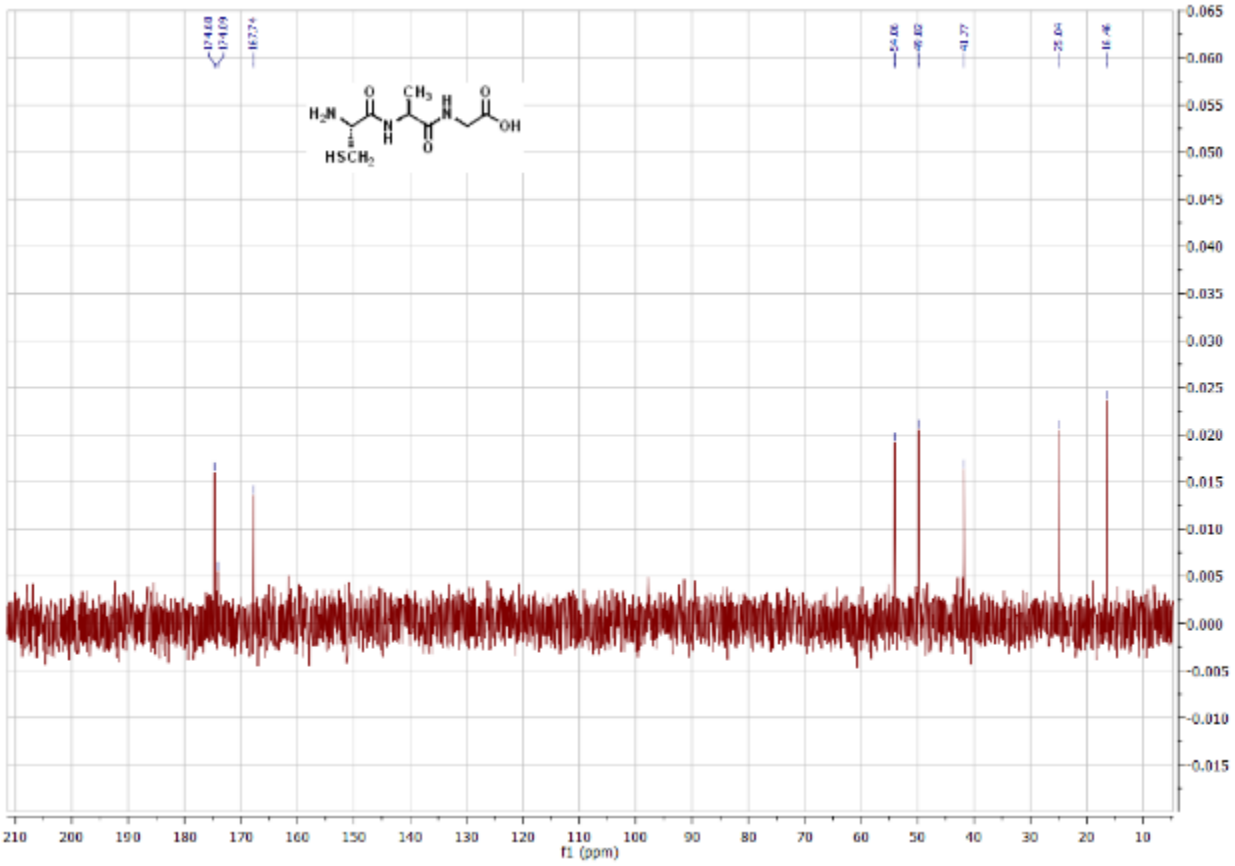


Fig. S13 ^{13}C NMR spectrum of **2.**

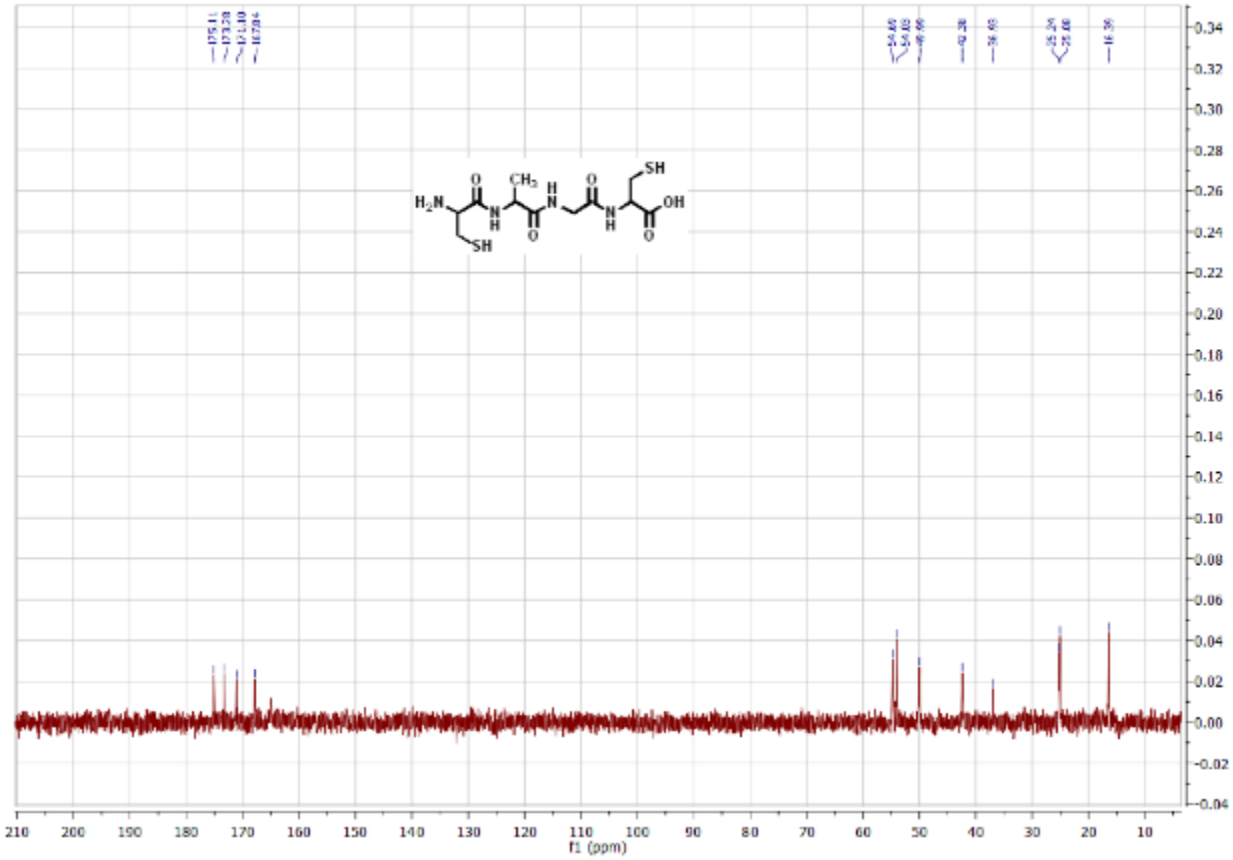


Fig. S14 ^{13}C NMR spectrum of **3.**

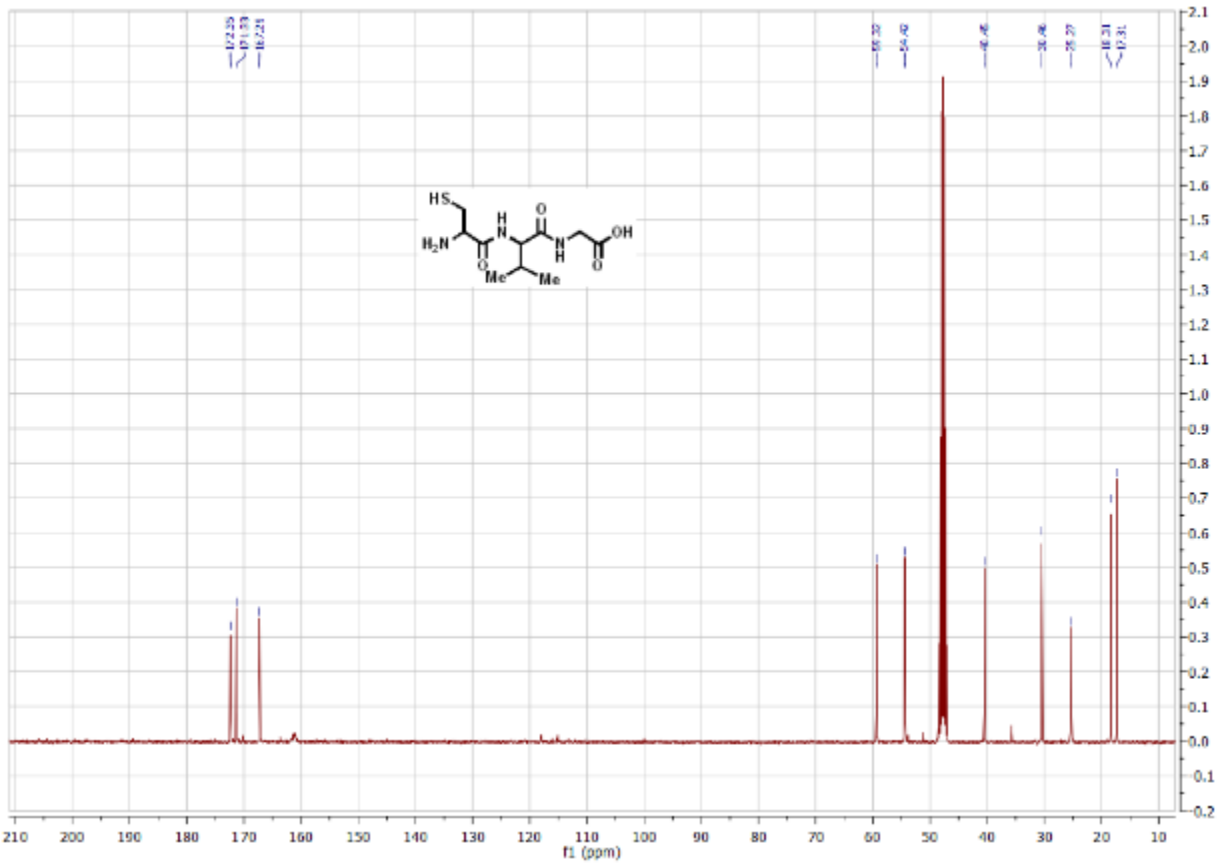


Fig. S15^{13}C NMR spectrum of **4.**

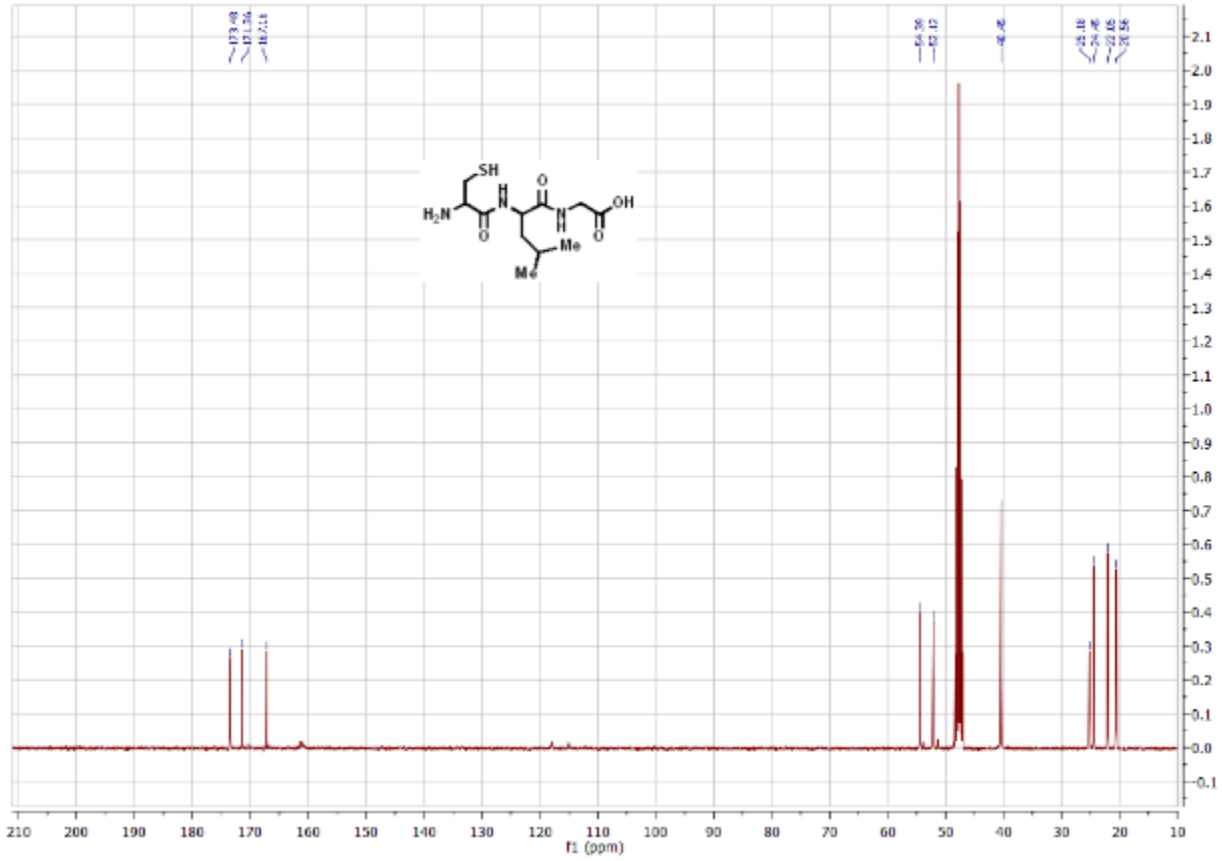


Fig. S16^{13}C NMR spectrum of **5.**

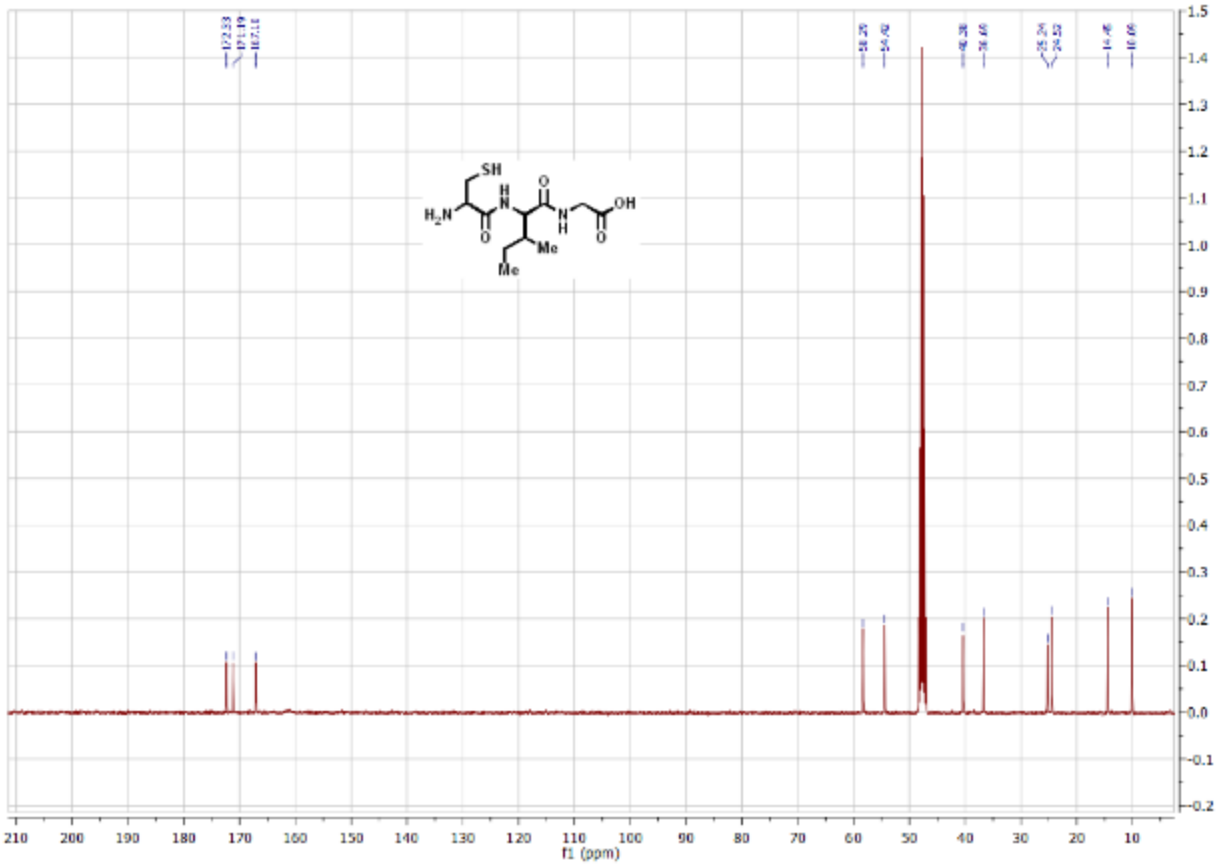


Fig. S17^{13}C NMR spectrum of **6.**

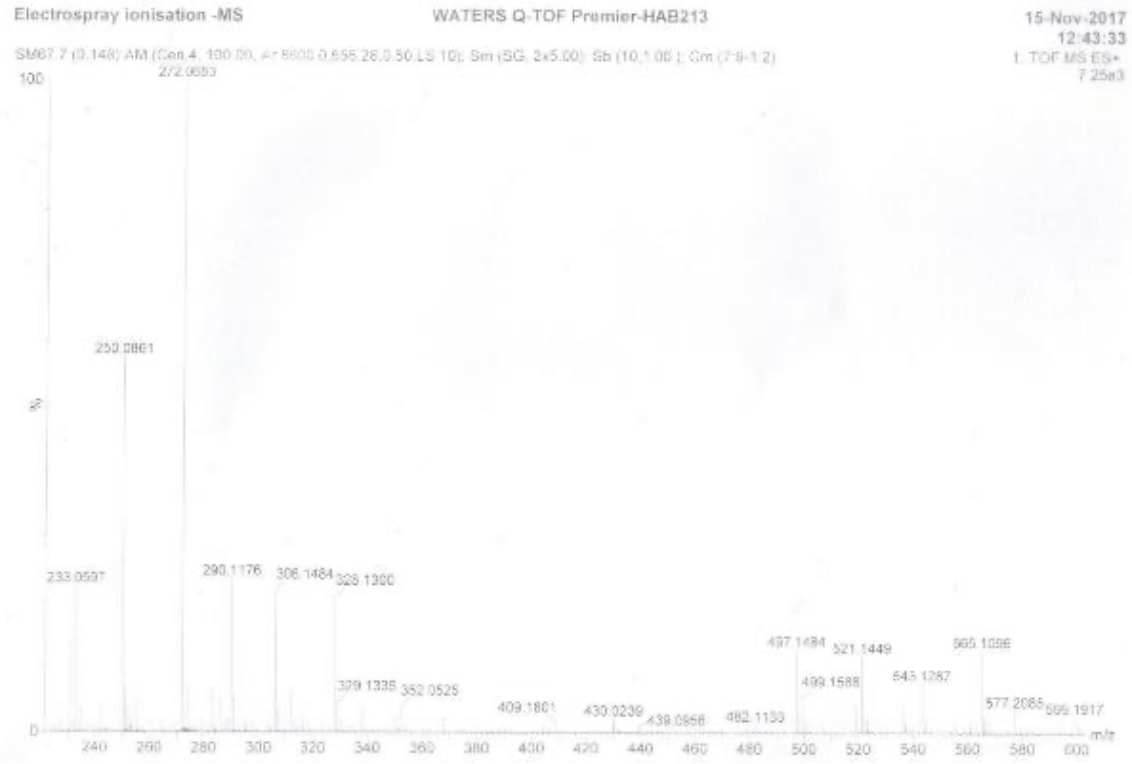


Fig. S18ESI-MS of **1.**

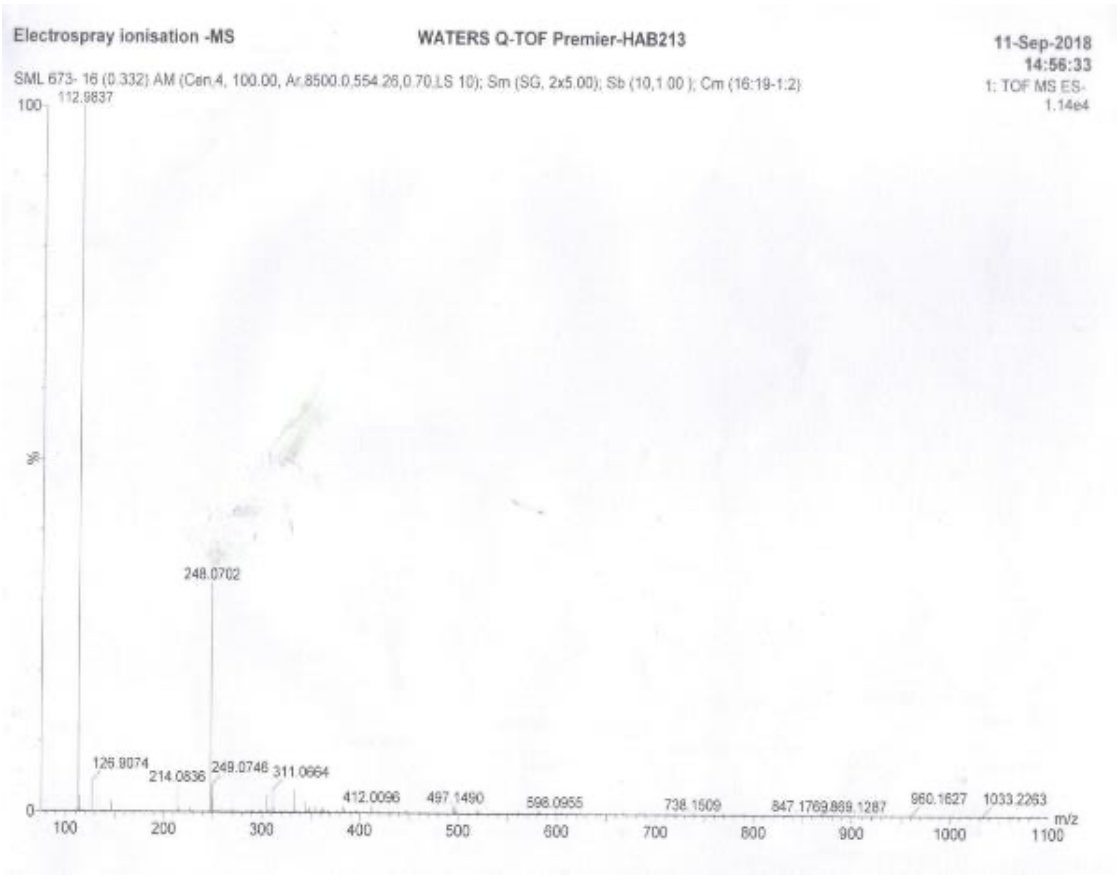


Fig. S19ESI-MS of**2.**

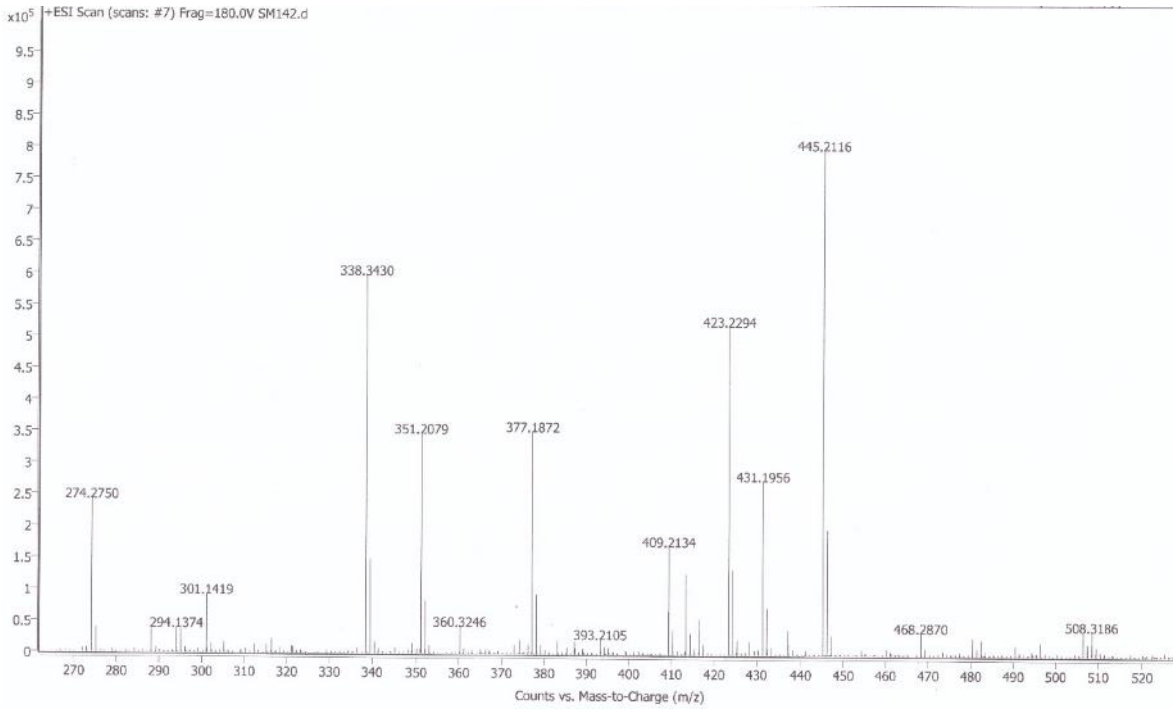


Fig. S20ESI-MS of **3.**

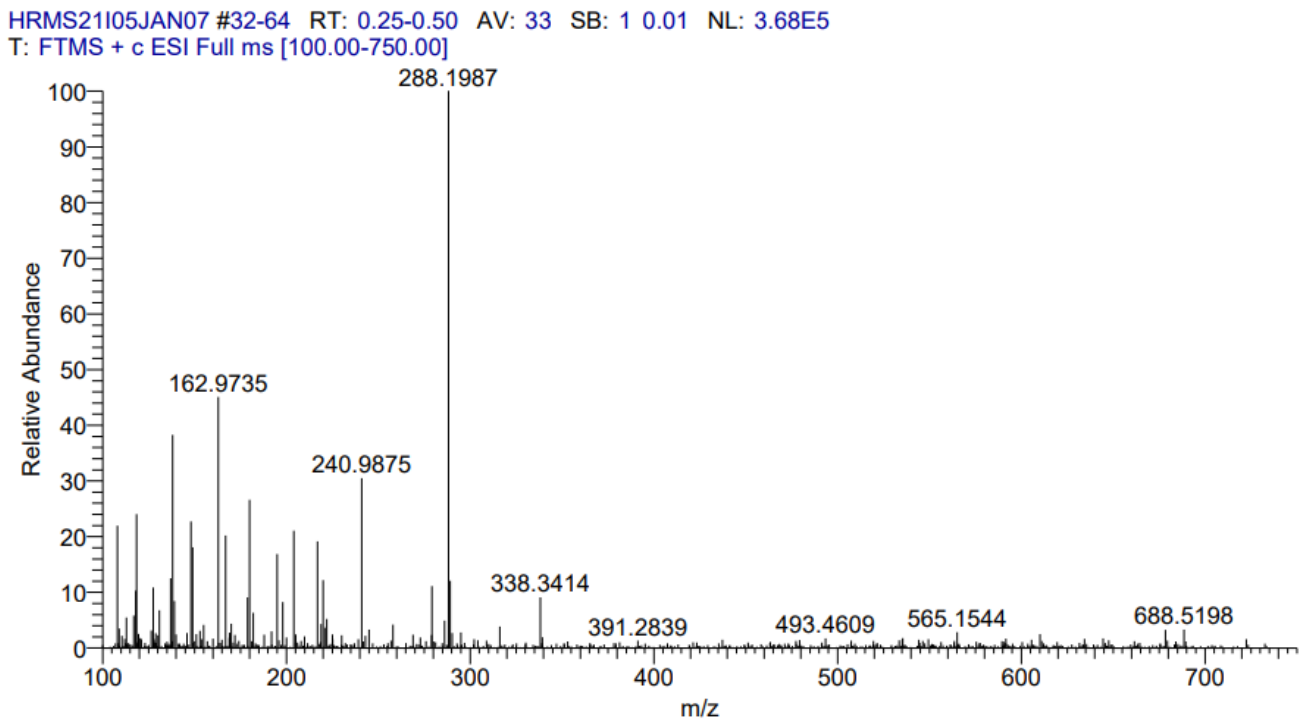


Fig. S21 ESI-MS of **4.**

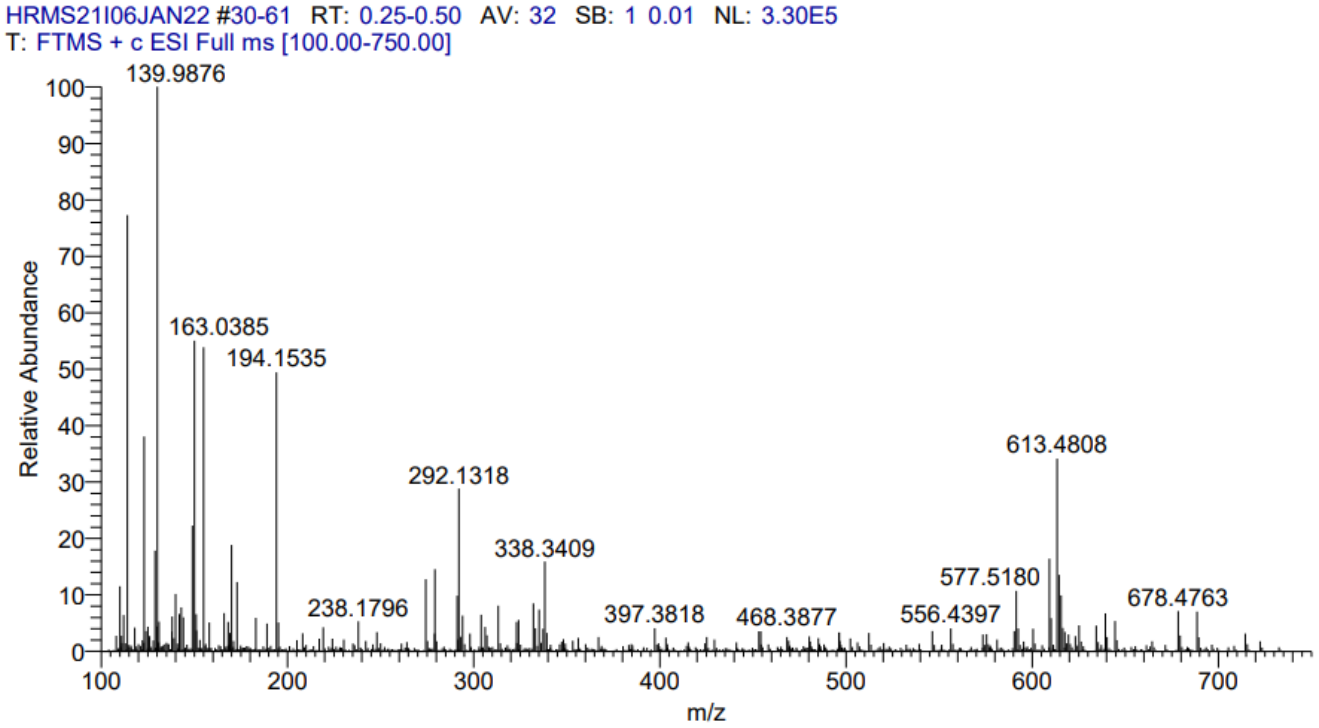


Fig. S22ESI-MS of **5.**

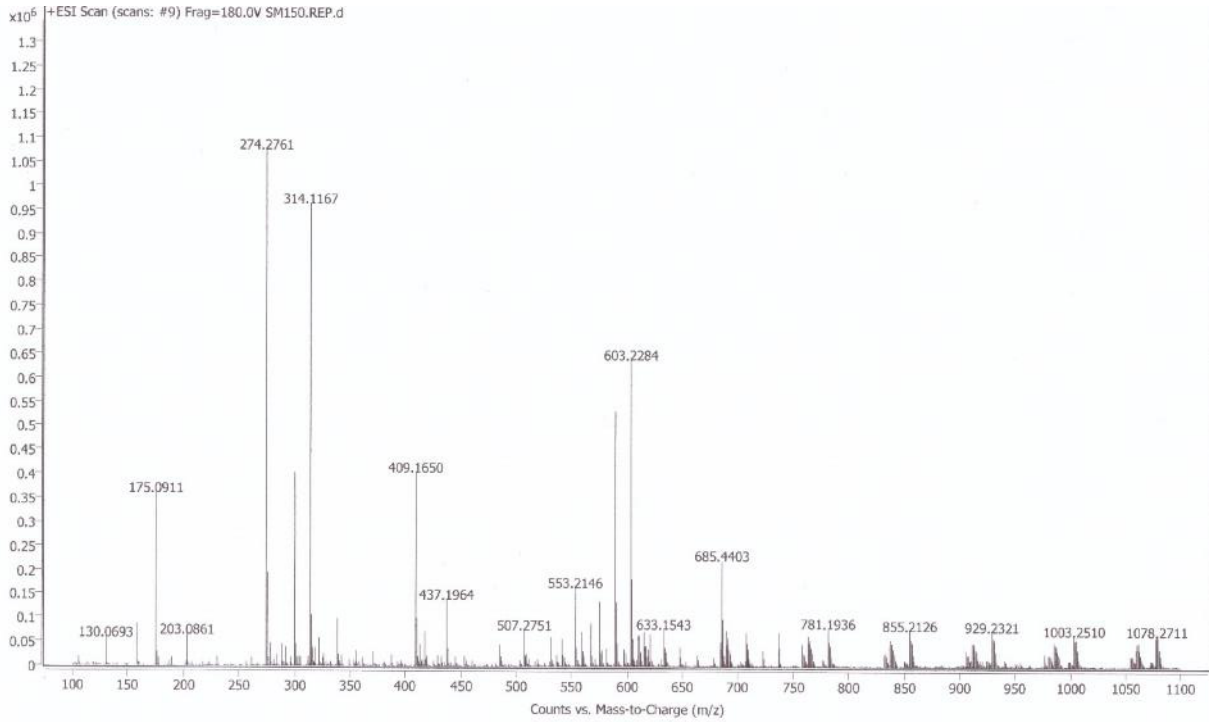


Fig. S23ESI-MS of **6.**

Table S2. List of primers used

S No.	**Primer Name**	**Primer Sequence**
1	GAPDH	**Forward primer** ACACAGTAGACTCCACG **Reverse primer** TCCAGTATGACTCCACTCAG
2	IL-12	**Forward primer** TTGATGGCCTGGAACTCTGT **Reverse primer** TTCCACAACAAGAGGGAGCT
3	IFN-γ	**Forward primer** TGAACGCTACACACTGCATC **Reverse primer** TCCTTTTGCCAGTTCCTCCA

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3 G. F. Späth and S. M. Beverley, *Exp. Parasitol.*, 2001, **99**, 97–103.

4 C. L. Yadav, K. K. Manar, M. K. Yadav, N. Tiwari, R. K. Singh, M. G. B. Drew and N. Singh, *J. Mol. Struct.*, 2018, **1160**, 488–496.

Author Contributions

SV and RKS conceptualized and supervised the study; SS, RKS, and SV designed and synthesized the molecules, analyzed the results and wrote manuscript. AA and RS performed the cell culture experiments and analyzed the results.