Synthesis, Profiling and In Vivo Evaluation of Cyclopeptides Containing N-Methyl Amino Acids as Antiplasmodial Agents

Catherine Fagundez,[1] Diver Sellanes,[1] Stella Peña,[1] Laura Scarone,[1] Anna C. C. Aguiar,[2] Juliana O. de Souza,[2] Rafael V. C. Guido,[2] Lindsay Stewart,[3] Vanessa Yardley,[3] Sabine Ottilie,[4] Elizabeth Winzeler,[4] Francisco J. Gamo,[5] Laura Sanz,[5] Gloria Serra[1]*

[1]Laboratorio de Química Farmacéutica, (DQO), Facultad de Química, Universidad de la República, Gral. Flores 2124, Montevideo, CP 11800, Uruguay, [2] Instituto de Física de São Carlos, Universidade de São Paulo, Brazil,[3] Faculty of Infectious & Tropical Disease, LSHTM, Keppel Street, London, WC1E 7HT, UK,[4] School of Medicine, University of California San Diego, La Jolla, CA, USA,[5] Diseases of the Developing World (DDW), Tres Cantos Medicines Development Campus, GlaxoSmithKline, Tres Cantos, Spain.

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Supporting Information

Table of contents

1. General Experimental Synthetic Methods……S3
2. SPPS and Solution Macrocyclization
   2.1. Loading of the 2-chlorotritylchloride resin. General procedure……S3
   2.2. SPPS general procedure for elongation of the peptide chain……S4
   2.3. General procedure for macrocyclization in solution phase……S4
3. SPPS and On- Resin Macrocyclization
   3.1. Loading of the 2-chlorotritylchloride resin. General procedure…… ……S5
   3.2. SPPS general procedure for elongation of the peptide chain……S5
   3.3. General procedure for on-resin macrocyclization……S6
4. Characterization Data of Products……S7-15
5. ^{1}H and ^{13}C NMR Spectra and Chromatograms for Compounds… …… ……S16-33
6. Determination of Physicochemicals Properties
   6.1 Determination of Solubility and Stability……34-35
   6.2 Determination of Log P……S35-36

6.3 PAMPA trans-membrane permeability determination……S36-37

7. Biological Evaluation Procedures

7.1 Procedure for *in vitro* evaluation against *Plasmodium falciparum*……S38

7.2 Procedure for *in vitro* cytotoxicity against HepG2 cells and murine macrophages……S39

7.3 *In vivo* assays against *P. berghei*……S40

7.4 Procedure for *in vivo* evaluation of plasma pharmacokinetic properties for compound **3**……S40-42

1. General Experimental Methods:

Optical rotation was measured using a Kruss Optronic GmbH P8000 polarimeter with a 0.5 mL cell. IR spectra were recorded on a Shimadzu FTIR 8101A spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Advance DPX- 400. Chemical shifts are related to TMS as an internal standard. High resolution mass spectra (HRMS) were obtained on a micro Q-TOF (ESI) (Bruker Daltonics) and LTQ-FT Ultra (NanoESI) (Thermo Scientific). Flash column chromatography was carried out with Silica gel 60 (J.T. Baker, 40 μm average particle diameter). All solution reactions and chromatographic separations were monitored by TLC, conducted on 0.25 mm Silica gel plastic sheets (Macherey/Nagel, Polygram_ SIL G/UV 254). TLC plates were analyzed under 254 nm UV light, iodine vapor, p-hydroxybenzaldehyde spray or ninhydrine spray. Yields are reported for chromatographically and spectroscopically (^{1}H and ^{13}C NMR) pure compounds.

All solid phase reactions were monitored by analytical HPLC on a Shimadzu (LC-10AT Pump) equipped with a Waters μBondapak™ C18 column (150 x 4.6 mm, 5μm) and a SPD20Aprominence UV/Vis detector.

All solvents were purified according to literature procedures.[1] All reactions were carried out in dry, freshly distilled solvents under anhydrous conditions unless otherwise stated.

2. SPPS and Solution Macrocyclization

2.1 Loading of the 2-chlorotritylchloride resin. General procedure.

The synthesis was done in a plastic syringe equipped with Teflon filters. The 2-chlorotritylchloride resin (100-300 mesh, 1.20 mmol/g) was swelled in CH_2Cl_2 (3 × 30 sec). A solution of the first protected aminoacid (Fmoc-AA-OH 2.0 eq) in CH_2Cl_2 and DIPEA (3.0 eq) were incorporated as a coupling system and were gently shaken for 10 min, then an extra 7.0 eq. of DIPEA was added and shaking was continued for 45 min. MeOH (0.8 mL/ g of resin) was added in order to cap unreacted functional groups on the resin; the mixture was then shaken for 10 min. The resin was filtered, and then washed with CH_2Cl_2 (x3), DMF (x3) and CH_2Cl_2 (x3). The loading capacity was estimated by Fmoc determination after the first residue attachment.

[1] Perrin, D. D. ; Armarego, W. L. F. "Purification of Laboratory Chemicals", 3th Ed. Pergamon Press, Oxford, 1988.

Fmoc-Gly-2-chlorotritylchloride Resin. Fmoc-Gly-OH was coupled to the 2-chlorotritylchloride Resin following the loading procedure. A loading of 1.20 mmo/g was calculated.

Fmoc-Cys(Trt)-2-chlorotritylchloride Resin. Fmoc-Cys(Trt)-OH was coupled to the 2-chlorotritylchloride Resin following the loading procedure. A loading of 1.12 mmo/g was calculated.

2.2 SPPS general procedure for elongation of the peptide chain.

The Fmoc-AA-2-chlorotritylchloride resin was shaken with 20% piperidine in DMF (2 × 5 min and 1 x 10 min), in order to deprotect N-terminus. The resin was then washed with DMF (x3), CH_2Cl_2 (x3) and DMF (x3). A solution of Fmoc-AA-OH (3 eq.) and DIPEA (6 eq.) in DMF was added to the resin, followed by a solution of HBTU or HCTU (2.9 eq) in DMF. If the amino acid is Fmoc-Cys(Trt)-OH, a solution of it (3 eq.), DIC (2.9 eq.) and Cl-HOBt (2.9 eq) in DMF was added to the resin. The mixture was stirred for 90 min. After the coupling was completed, the resin was washed with DMF (×3) and CH_2Cl_2 (x3). Deprotection and coupling cycles were repeated with the appropriate amino acids to provide the desired compound. Completion of the coupling was monitored by analytical HPLC. The peptide was cleaved from the resin by treatment with 1% TFA in CH_2Cl_2 for 2-3 minutes at room temperature followed by filtration and collection of the filtrate in MeOH. The treatment was repeated three times and then the resin washed with CH_2Cl_2 (x5). Solvents were removed *in vacuo* to obtain the crude peptide.

2.3 General procedure for macrocyclization in solution phase.

Macrocyclization reaction was performed in diluted conditions (1-5 mM) using HBTU or HATU (1.5 eq.), DIPEA (3 eq.), 4-DMAP (catalytic) in dried CH_2Cl_2 at room temperature during 1-3 days. The reaction mixture was washed with HCl 5% and then with saturated aqueous $NaHCO_3$, dried over MgSO4, filtered and concentrated in vacuo. The crude was purified by flash chromatography to obtain the macrocycle.

3. SPPS and On- Resin Macrocyclization

3.1 Loading of the 2-chlorotritylchloride resin. General procedure.

The synthesis was done in a plastic syringe equipped with Teflon filters. The 2-chlorotritylchloride resin (100-300 mesh, 1.20 mmol/g) was swelled in CH_2Cl_2 (3 × 30 sec). A solution of the first protected aminoacid (Fmoc-AA-OH 2.0 eq) in CH_2Cl_2 and DIPEA (3.0 eq) were incorporated as a coupling system and were gently shaken for 10 min, then an extra 7.0 eq. of DIPEA was added and shaking was continued for 45 min. MeOH (0.8 mL/ g of resin) was then added in order to cap unreacted functional groups on the resin; the mixture was then shaken for 10 min. The resin was filtered, and then washed with CH_2Cl_2 (x3), DMF (x3) and CH_2Cl_2 (x3). The loading capacity was estimated by Fmoc determination after the first residue attachment.

Fmoc-L-Glu(αOAllyl)-2-chlorotritylchloride Resin. Fmoc-L-Glu(αOAllyl)-OH was coupled to the 2-chlorotritylchloride Resin following the loading procedure. A loading of 1.10 mmol/g was calculated.

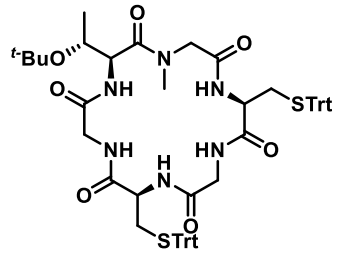
3.2 SPPS general procedure for elongation of the peptide chain:

The Fmoc-L-Glu(αOAllyl)-2-chlorotritylchloride resin was shaken with 20% piperidine in DMF (2 × 5 min and 1 x 10 min), in order to deprotecting N-terminus. The resin was then washed with DMF (x3), CH_2Cl_2 (x3) and DMF (x3). A solution of Fmoc-AA-OH (3 eq.) and DIPEA (6 eq.) in DMF was added to the resin, followed by a solution of HBTU or HCTU (2.9 eq) in DMF; or, if the amino acid is Fmoc-Cys(Trt)-OH, a solution of Fmoc-AA-OH (3 eq.), DIC (2.9 eq.) and Cl-HOBt (2.9 eq) in DMF was added to the resin. The mixture was stirred for 90 min. After the coupling was completed, the resin was washed with DMF (x3) and CH_2Cl_2 (x3). Deprotection and coupling cycles were repeated with the appropriate amino acids to provide the desired compound. The peptide protected at both ends, was deprotected with $[Pd(PPh_3)_4]$ (0.5 eq) in a solution of piperidine 10% in THF for 3 hs. After the deprotection was completed, the resin was filtered and then washed with DMF (x3), CH_2Cl_2 (x3). The resin was stirred for 30 min with a solution of sodium diethyldithiocarbamate 5% (w/v) and DIPEA 5% in DMF. The resin was filtered and then washed with DMF, CH_2Cl_2 and MeOH exhaustively.

3.3 General procedure for on-resin macrocyclization.

Macrocyclization reaction was performed on-resin using DIC (4.0 eq.), Cl-HOBt (4.0 eq.), 4-DMAP (catalytic) in dried DMF:CH_2Cl_2 (8:2) at room temperature overnight. After the macrocyclization was completed, the resin was filtered and then washed with DMF (x3), CH_2Cl_2 (x3). The macrocycle was cleaved from the resin by treatment with 1% TFA in CH_2Cl_2 for 2-3 minutes at room temperature followed by filtration and collection of the filtrate in MeOH. The treatment was repeated three times and then the resin was washed with CH_2Cl_2 (x5). Solvents were removed *in vacuo* to obtain the crude macrocycle.

4. Characterization data of products

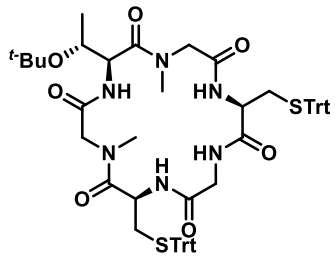


2

The trifluoroacetate salt of NH_2-*L*-Thr(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-Gly-OH was obtained following the general SPPS procedure, using HCTU/DIPEA for Fmoc-AA-OH coupling after NHMe-Gly, yielded 750 mg (89% yield) as a white solid. The purity (98 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 9.05 min).

Macrocyclization reaction was performed following the general procedure (dilution 5mM, 3 days), starting from the trifluoroacetate salt of the amino acid trifluoroacetato NH_2-*L*-Thr(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-Gly-OH (660 mg, 0,566 mmol), using HATU as coupling agent. Further purification by flash chromatography, rendered the desired macrocycle (451mg, 0,436 mmol) in 77% yield.

***Cyclo*-[*L*-Thr(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-Gly] (2):** White solid (77%). **Rf**= 0.5 (EtOAc). **$[\alpha]_D^{25}$=** +20.5 (*c* 1.9, DCM). **^{1}H NMR (400 MHz, $CDCl_3$)** δ (ppm): 0.99 (d, *J*=6.3, Hz, 3H), 1.23 (s, 9H), 2.47-2.56 (m, 1H), 2.65-2.72 (m, 1H), 2.76-2.84 (m, 1H), 3.07-3.15 (m, 1H), 3.19-3.26 (m, 1H), 3.28 (s, 3H), 3.46-3.55 (m, 1H), 3.57-3.66 (m, 1H), 3.71-3.74 (m, 1H), 3.91-4.03 (m, 2H), 4.04-4.17 (m, 2H), 4.35-4.43 (m, 1H), 4.67 (t, *J*=5.4 Hz, 1H), 6.09-6.17 (m, 1H), 6.18-6.26 (m, 1H), 6.67-6.76 (m, 1H), 7.08-7.16 (m, 1H), 7.19-7.37 (m, 19H), 7.38-7.50 (m, 12H). **^{13}C NMR (100 MHz, ($CDCl_3$)** δ(ppm): 18.5, 28.2 (3C), 31.8, 32.8, 39.2, 43.0, 43.1, 52.2, 53.3, 54.1, 54.7, 67.4 (2C), 67.7, 75.1, 127.0 (6C), 128.1 (12C), 129.5 (12C), 144.3 (3C), 144.4 (3C), 168.7, 168.9, 169.4, 170.4, 170.5, 170.8. **HRMS** *m/z* calc. for $C_{59}H_{64}N_6NaO_7S_2$ ($[M+Na]^+$) 1055.4170, found 1055.4148.

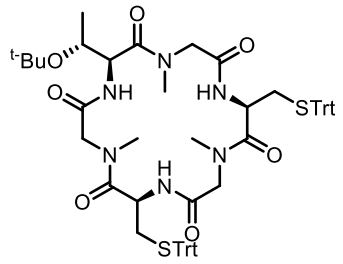


3

The trifluoroacetate salt of NHMe-Gly-*L*-Thr(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-OH was obtained following the general SPPS procedure, using HCTU/DIPEA for Fmoc-AA-OH coupling after NHMe-Gly, yielded 502 mg (90% yield) as a white solid. The purity (95 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 9.24 min).

Macrocyclization reaction was performed following the general procedure (dilution 5mM, 1 day), starting from the trifluoroacetate salt of the amino acid trifluoroacetato NH_2-NMe-Gly-*L*-Thr(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-OH (350 mg, 0,270 mmol), using HATU as coupling agent. Further purification by flash chromatography, rendered the desired macrocycle (120mg, 0,115 mmol) in 42% yield.

***Cyclo*-[NMe-Gly-*L*-Thr(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)] (3):** White solid (42%). **Rf**= 0.5 (EtOAc). **$[\alpha]_D^{25}$=** +85.9 (*c* 1.43, DCM). **^{1}H NMR (400 MHz, $(CD_3)_2CO$)** δ (ppm): 1.13 (s, 9H), 1.21 (d, *J*=5.8 Hz, 3H), 2.58-2.70 (m, 1H), 2.65-2.72 (m, 3H), 2.95 (s, 3H), 3.34 (s, 3H), 3.60-3.73 (m, 2H), 3.76-3.85 (m, 1H), 3.97-4.04 (m, 1H), 4.14-4.23 (m, 2H), 4.38-4.48 (m, 2H), 4.54-4.62 (m, 2H), 7.24-7.45 (m, 30H), 7.52-7.58 (m, 1H), 7.63-7.68 (m, 1H), 7.90-7.95 (m, 1H), 7.96-8.02 (m, 1H). **^{13}C NMR (100 MHz, $(CD_3)_2CO$)** δ(ppm): 20.3, 27.2 (3C), 31.6, 32.8, 37.9 (2C), 42.4, 51.0, 52.1, 52.3, 53.5, 56.6, 65.9, 66.9, 67.2, 74.2, 127.0 (3C), 127.1 (3C), 128.1 (12C), 129.4 (6C), 129.5 (6C), 144.3 (6C), 165.1, 170.2, 170.5, 171.5, 171.7, 172.0. **HRMS** *m/z* calc. for $C_{60}H_{66}N_6NaO_7S_2$ ($[M+Na]^+$) 1069.4332, found 1069.4327.

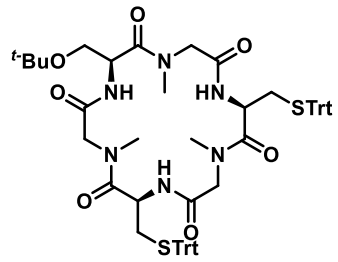


4

The trifluoroacetate salt of H_2N-L-Thr(^{t}Bu)-NMeGly-L-Cys(Trt)-NMeGly-L-Cys(Trt)-NMeGly-OH was obtained following the general SPPS procedure, using HCTU/DIPEA for Fmoc-AA-OH coupling after NHMe-Gly, yielded 124 mg (75% yield) as a white solid. The purity (97 %) was determined by HPLC (linear gradient: 8 to 100% acetonitrile in H_2O/ 0.1% TFA over 20 min; flow rate = 1.0 mL/min; t_R = 13.5 min).

Macrocyclization reaction was performed following the general procedure (dilution 5 mM, 2 days), starting from the trifluoroacetate salt of H_2N-L-Thr(^{t}Bu)-NMeGly-L-Cys(Trt)-NMeGly-L-Cys(Trt)-NMeGly-OH (78 mg, 0.065 mmol), using HBTU as coupling agent. Further purification by flash chromatography, rendered the desired macrocycle (26 mg, 0.023 mmol) in 35% yield.

***Cyclo*-[L-Thr(^{t}Bu)-NMeGly-L-Cys(Trt)-NMeGly-L-Cys(Trt)-NMeGly] (4).** White Solid (35%), **Rf** = 0.55 (AcOEt:EP, 4:1). **$[\alpha]_D^{25}$=** +41.7 (*c* 1.1, DCM). **^{1}H NMR (400 MHz, $CDCl_3$)** δ (ppm) 1.02 (d, *J* = 6.4 Hz, 3H), 1.24 (s, 9H), 2.42– 2.53 (m, 2H), 2.60 – 2.71 (m, 2H), 2.80 (s, 3H), 2.82 (s, 3H), 2.95– 3.16 (m, 3H), 3.27 (s, 3H), 3.93 (dd, *J* = 6.4 Hz, *J* = 3.8 Hz, 1H), 4.54 (d, *J* = 13.6 Hz, 1H), 4.63 (d, *J* = 13.2 Hz, 1H), 4.64 (d, *J* = 13.4 Hz, 1H), 4.72 – 4.80 (m, 3H), 7.14 – 7.63 (m, 30H), 7.54 – 7.63 (m, 7.67, 2H), 7.67 (d, *J* = 6.8 Hz, 1H). **^{13}C NMR (100 MHz, $CDCl_3$)** δ (ppm) 18.0, 28.0, 34.4, 36.6, 36.9, 38.4, 48.5, 53.3, 53.8, 54.0, 54.5, 66.9, 68.2, 75.5 , 126.8, 128.0, 129.6, 144.4, 167.1, 167.4, 167.6, 169.8, 170.2, 170.3. **HRMS** calc. for $C_{61}H_{68}N_6NaO_7S_2$ ($[M+Na]^+$) 1083.4489 found 1083.4447

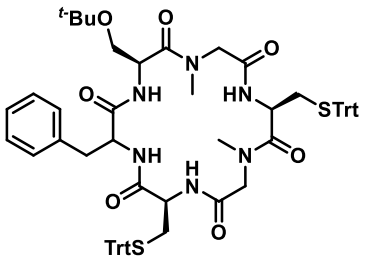


5

The trifluoroacetate salt of NHMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Ser(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-OH was obtained following the general SPPS procedure, using HCTU/DIPEA for Fmoc-AA-OH coupling after NHMe-Gly, yielded 733 mg (87% yield) as a white solid. The purity (98 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 9.13 min).

Macrocyclization reaction was performed following the general procedure (dilution 5mM, 1 days), starting from the trifluoroacetate salt of the amino acid NH_2-NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Ser(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-*OH* (550 mg, 0.430 mmol), using HATU as coupling agent. Further purification by flash chromatography, rendered the desired macrocycle (334 mg, 0.319 mmol) in 74% yield.

***Cyclo*-[NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Ser(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)] (5):** White solid (74%). **Rf**= 0.5 (EtOAc). **$[\alpha]_D^{25}$=** +74.0 (*c* 0.99, DCM). **^{1}H NMR (400 MHz, $(CD_3)_2CO$)** δ (ppm): 1.14 (s, 9H), 2.46-2.60 (m, 1H), 2.64-2.73 (m, 1H), 2.75-3.07 (m, 10H), 3.11-3.29 (m, 4H), 3.33-3.47 (m, 1H), 3.51-3.72 (m, 1H), 4.41-4.60 (m, 3H), 4.65-4.81 (m, 2H), 4.83-4.94 (m, 1H), 7.02-7.59 (m, 33H). **^{13}C NMR (100 MHz, $(CD_3)_2CO$)** δ(ppm): 27.3 (3C) 34.2, 37.0, 38.6 (3C), 48.6 (2C), 50.0, 53.4 (2C), 53.9, 63.0, 67.1 (2C), 73.8, 126.9 (6C), 128.0 (12C), 129.7 (12C),144.4 (6C), 167.4, 167.5, 167.7, 170.5 (2C), 170.8. **HRMS** *m/z* calc. para $C_{60}H_{66}N_6NaO_7S_2$ ($[M+Na]^+$) 1069.4325, found 1069.4327.

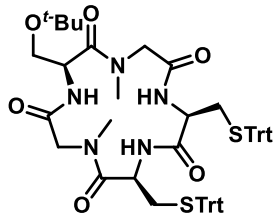


6

The trifluoroacetate salt of NHMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Cys(Trt)-*L*-Phe-*L*-Ser(^{t}Bu)-OH was obtained following the general SPPS procedure, using HCTU/DIPEA for Fmoc-AA-OH coupling after NHMe-Gly, yielded 450 mg (82% yield) as a white solid. The purity (98 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 9.51 min).

Macrocyclization reaction was performed following the general procedure (dilution 5mM, 2 days), starting from the trifluoroacetate salt of the amino acid NHMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Cys(Trt)-*L*-Phe-*L*-Ser(^{t}Bu)-OH (450 mg, 0.358 mmol), using HATU as coupling agent. Further purification by flash chromatography, rendered the desired macrocycle (231mg, 0.21 mmol) in 57% yield.

***Cyclo*-[NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Cys(Trt)-*L*-Phe-*L*-Ser(^{t}Bu)] (6):** White solid (57%). **Rf**= 0.4 (EtOAc). **$[\alpha]_D^{25}$=** -24.3 (*c* 2.40, DCM). **^{1}H NMR (400 MHz, $(CD_3)_2CO$)** δ (ppm): 1.06 (s, 9H), 2.47-2.60 (m, 3H), 2.86-3.02 (m, 2H), 2.99 (s, 3H), 3.07-3.23 (m, 2H), 3.18 (s, 3H), 3.39-3.50 (m, 2H), 3.65 (dd, *J*=5.0, 9.3 Hz, 1H), 4.07-4.15 (m, 1H), 4.16-4.24 (m, 1H), 4.30 (d, *J*= 14.4 Hz, 1H), 4.50 (dd, *J*= 8.1Hz, 1H), 4.62-4.70 (m, 1H), 4.90 (d, *J*= 16.7 Hz, 1H), 7.11-7.47 (m, 35H), 7.53 (d, *J*= 4.9 Hz, 1H), 7.69 (d, *J*= 9.1 Hz, 1H), 7.76 (d, *J*= 8.2 Hz, 1H), 7.88 (d, *J*= 9.0 Hz, 1H). **^{13}C NMR (100 MHz, $(CD_3)_2CO$)** δ(ppm): 26.8 (3C), 32.8, 32.9, 35.6, 36.4, 38.4, 47.44, 51.2, 52.8, 53.3, 53.4, 53.6, 61.8, 66.5, 66.7, 72.7, 126.3, 126.8 (3C), 126.8 (3C), 128.0 (12C), 128.2 (2C), 129.4 (2C), 129.5 (6C), 129.6 (6C), 137.7, 144.6 (3C), 144.9 (3C), 167.3, 168.8, 169.6, 169.9, 171.0, 171.5. **HRMS** *m/z* calc. para $C_{66}H_{70}N_6NaO_7S_2$ ($[M+Na]^+$) 1146.4640, found 1146.4666.

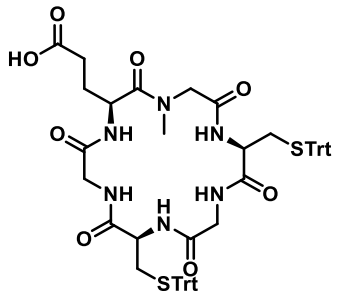


7

The trifluoroacetate salt of NHMe-Gly-*L*-Ser(*t*-Bu)-NMe-Gly-*L*-Cys(Trt)-*L*-Cys(Trt)-OH was obtained following the general SPPS procedure, using HCTU/DIPEA for Fmoc-AA-OH coupling after NHMe-Gly, yielded 240 mg (67% yield) as a white solid. The purity (95 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 9.18 min).

Macrocyclization reaction was performed following the general procedure (dilution 5 mM, 1 day), starting from the trifluoroacetate salt of the amino acid trifluoroacetato NHMe-Gly-*L*-Ser(*t*-Bu)-NMe-Gly-*L*-Cys(Trt)-*L*-Cys(Trt)-OH (240 mg, 0,218 mmol), using HATU as coupling agent. Further purification by flash chromatography, rendered the desired macrocycle (125 mg, 0,128 mmol) in 59% yield.

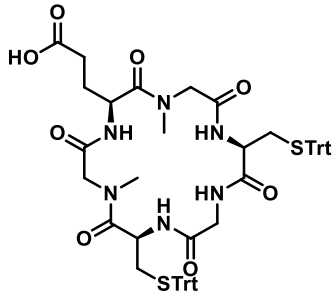
***Cyclo*-[NMe-Gly-*L*-Ser(*t*-Bu)-NMe-Gly-*L*-Cys(Trt)-*L*-Cys(Trt)] (7):** White solid (59%). **Rf**= 0.5 (EtOAc). **$[\alpha]_D^{25}$=** -12.3 (*c* 1.66, DCM). **^{1}H NMR (400 MHz, $(CD_3)_2CO$)** δ (ppm): 1.14 (s, 9H), 2.18-2.27 (m, 1H), 2.49 (dd, *J*=4.0, 12.5 Hz, 1H), 2.65-2.82 (m, 5H), 2.94 (d, *J*=13.6 Hz, 1H), 3.28 (s, 3H), 3.38-3.50 (m, 3H), 4.15-4.26 (m, 2H), 4.63-4.72 (m, 1H), 4.82 (d, *J*=13.6 Hz, 1H), 4.91-4.94 (m, 1H), 7.06 (d, *J*=9.9 Hz, 1H), 7.17-7.35 (m, 18H), 7.35-7.43 (m, 12H), 7.54 (d, *J*=9.2 Hz, 1H), 7.64 (d, *J*=7.9 Hz, 1H). **^{13}C NMR (100 MHz, $(CD_3)_2CO$)** δ(ppm): 26.6 (3C), 33.3, 33.8, 35.2, 38.2, 49.1, 49.5, 52.8, 53.3, 53.9, 62.4, 66.6 (2C), 78.4, 126.6 (3C), 126.8 (3C), 127.9 (6C), 128.0 (6C), 129.5 (6C), 129.6 (6C), 144.6 (3C), 145.1 (3C), 168.6, 168.9, 169.6, 169.8, 169.9, 173.0. **HRMS** *m/z* calc. para $C_{57}H_{61}N_5NaO_6S_2$ ($[M+Na]^+$) 998.3964, found 998.3955.



8

Cyclo-[NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-Gly-*L*-Glu] was obtained following the general SPPS and macrocyclation on-resin procedures, yielded 195 mg (81% yield) of the crude macrocycle as a yellow solid. The purity (64 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 10.65 min). Then the compound was purified (94%)

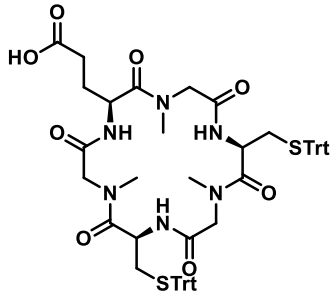
***Cyclo*-[NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-Gly-*L*-Glu] (8):** Yellow solid (44%). t_R = 10.65 min (linear gradient: 50 a 100% acetonitrile in H_2O/0.003M TFA over 20min; flow rate = 1.5 ml/min). **^{1}H NMR (400 MHz, $(CD_3)_2CO$)** δ (ppm): 1.77-1.93 (m, 2H), 2.28-2.43 (m, 2H), 2.61-2.79 (m, 4H), 3.30 (s, 3H), 3.51-3.68 (m, 2H), 3.79-3.96 (m, 3H), 3.96-4.10 (m, 1H), 4.18-4.34 (m, 1H), 4.35-4.45 (m, 1H), 4.81-4.90 (m, 1H), 7.15-7.51 (m, 36H). **^{13}C NMR (100 MHz, $(CD_3)_2CO$)** δ(ppm): 21.9, 28.6, 32.0, 33.2, 37.1, 42.1, 42.2, 48.7, 50.3, 52.6, 53.6, 66.6, 66.7, 126.8 (6C), 128.0 (12C), 129.5 (12C), 144.7 (6C), 168.5, 169.5 (2C), 169.7, 169.8, 169.9, 173.4. **(ESI-MS)** *m/z* calc. for $C_{56}H_{55}N_6O_8S_2$ ($[M-H]^-$) 1003.35, found 1003.04.



9

Cyclo-[NMe-Gly-L-Cys(Trt)-Gly-L-Cys(Trt)-NMe-Gly-L-Glu] was obtained following the general SPPS and macrocyclation on-resin procedures, yielded 206 mg (92% yield) of the crude macrocycle as a yellow solid. The purity (58 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 11.02 min). Then the compound was purified (90%)

***Cyclo*-[NMe-Gly-L-Cys(Trt)-Gly-L-Cys(Trt)-NMe-Gly-L-Glu] (9):** Yellow solid (44%). t_R = 11.02 min (linear gradient: 50 a 100% acetonitrile in H_2O/ 0.003M TFA over 20min; flujo = 1,5 ml / min). **^{1}H NMR (400 MHz, $(CD_3)_2CO$)** δ (ppm): 1.76-1.94 (m, 2H), 2.24-2.45 (m, 2H), 2.53-2.75 (m, 3H), 2.77-2.90 (m, 1H), 2.96 (s, 3H), 3.12-3.22 (m, 1H), 3.33 (s, 3H), 3.64-3.77 (m, 2H), 3.84--4.14 (m, 2H), 4.16-4.28 (m, 1H), 4.31-4.42 (m, 1H), 4.68-4.79 (m, 1H), 4.89-5.02 (m, 1H), 7.06-7.50 (m, 35H). **^{13}C NMR (100 MHz, $(CD_3)_2CO$)** δ(ppm): 26.9, 28.8, 32.7, 33.0, 36.3, 37.5, 42.1, 48.4, 51.4, 52.1, 52.7, 53.5, 66.6, 66.8, 126.8 (6C), 128.0 (12C), 129.5 (12C), 144.8 (6C), 167.6, 168.4, 168.7, 170.11, 171.6, 171.6, 173.3. **(ESI-MS)** *m/z* calc. for $C_{57}H_{57}N_6O_8S_2$ ($[M-H]^-$) 1017.37 found 1017.56



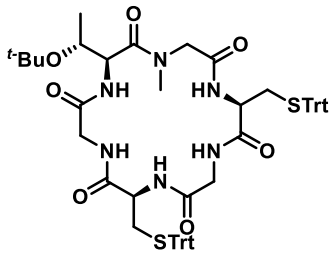
10

Cyclo-[NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Glu] was obtained following the general SPPS and macrocyclation on-resin procedures, yielded 412 mg (84% yield) of the crude macrocycle as a yellow solid. The purity (84 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile in H_2O/ 0.003M TFA over 10 min; flow rate = 1.5 mL/min; t_R = 9.40 min). Then the compound was purified (94%)

***Cyclo*-[NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Glu] (10):** Yellow solid (71%). t_R = 9.40 min (linear gradient: 50 a 100% acetonitrile in H_2O/0.003M TFA over 20min; flow rate = 1.5 ml/min). **^{1}H NMR (400 MHz, $(CD_3)_2CO$)** δ (ppm): 2.31-2.39 (m, 2H), 2.39-2.41 (m, 2H), 2.54-2.74 (m, 4H), 2.82-2.91 (m, 9H), 3.29-3.54 (m, 2H), 3.75-3.88 (m, 1H), 4.17-4.32 (m, 1H), 4.36-4.52 (m, 2H), 4.64-4.86 (m, 3H), 4.86-5.06 (m, 1H), 7.18-7.47 (m, 33H). **^{13}C NMR (100 MHz, $(CD_3)_2CO$)** δ(ppm): 22.7, 29.5, 35.0 (2C), 38.1, 47.4, 48.4 48.5, 51.3, 51.7 (2C), 66.2, 66.5, 127.1, 128.0, 128.2, 129.6, 144.7, 145.0, 169.9, 170.0 (2C), 170.6, 171.0, 172.0, 174.4. **(ESI-MS)** *m/z* calc. for $C_{58}H_{60}N_6NaO_8S_2$ ($[M+Na]^+$) 1055.38. found 1055.26; $C_{58}H_{61}N_6O_8S_2$ ($[M+H]^+$) 1033.40. found 1033.28.

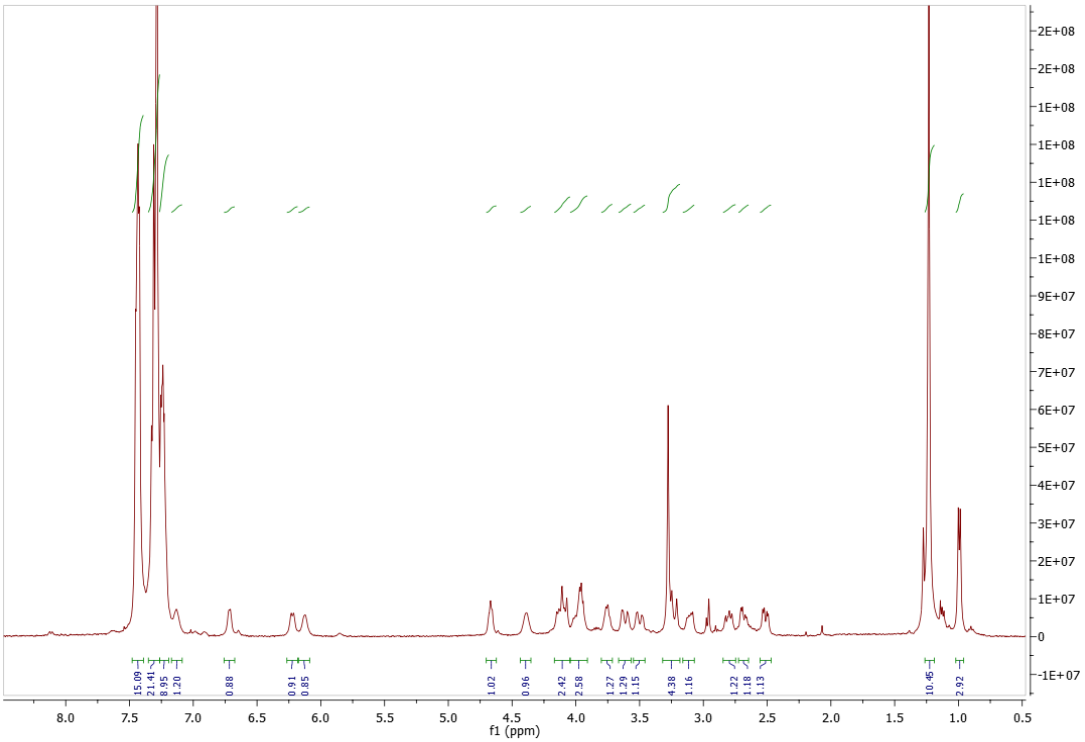
5. ^{1}H and ^{13}C NMR Spectra and Chromatograms for Compounds

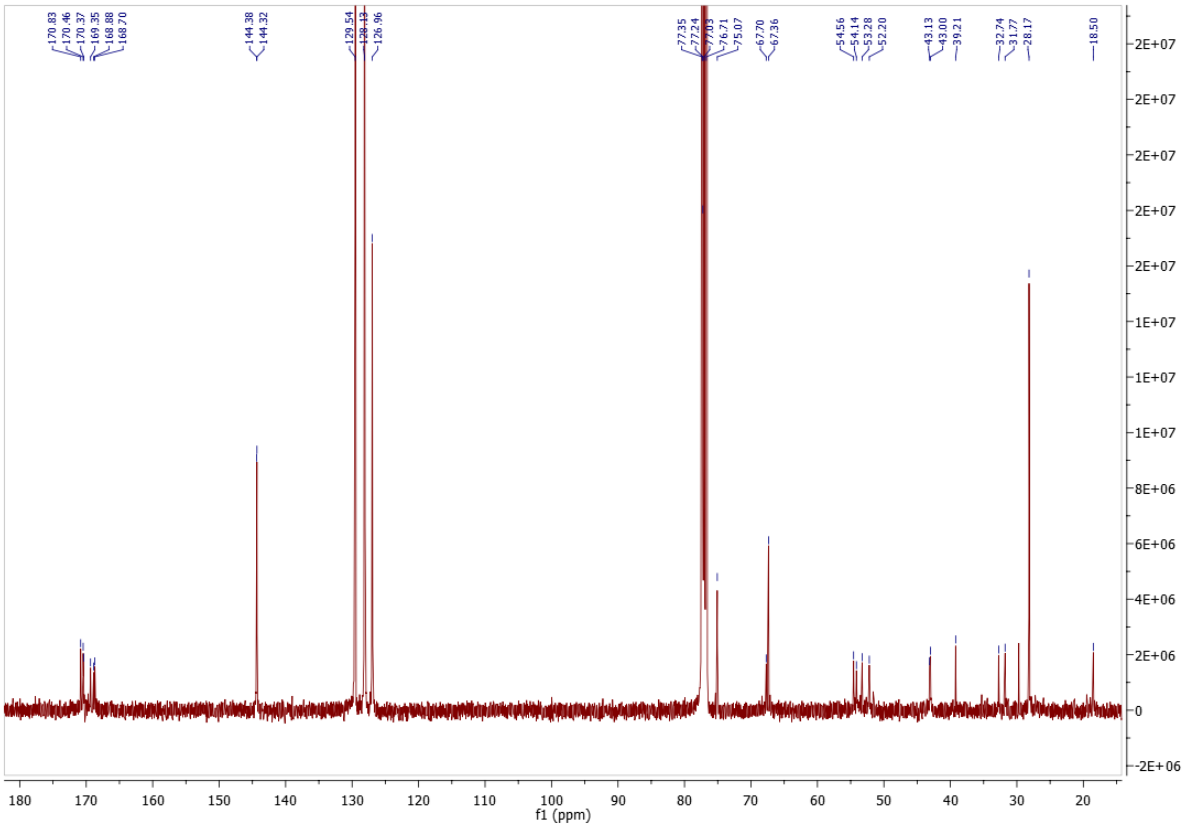
***Cyclo*-[*L*-Thr(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-Gly] (2)**

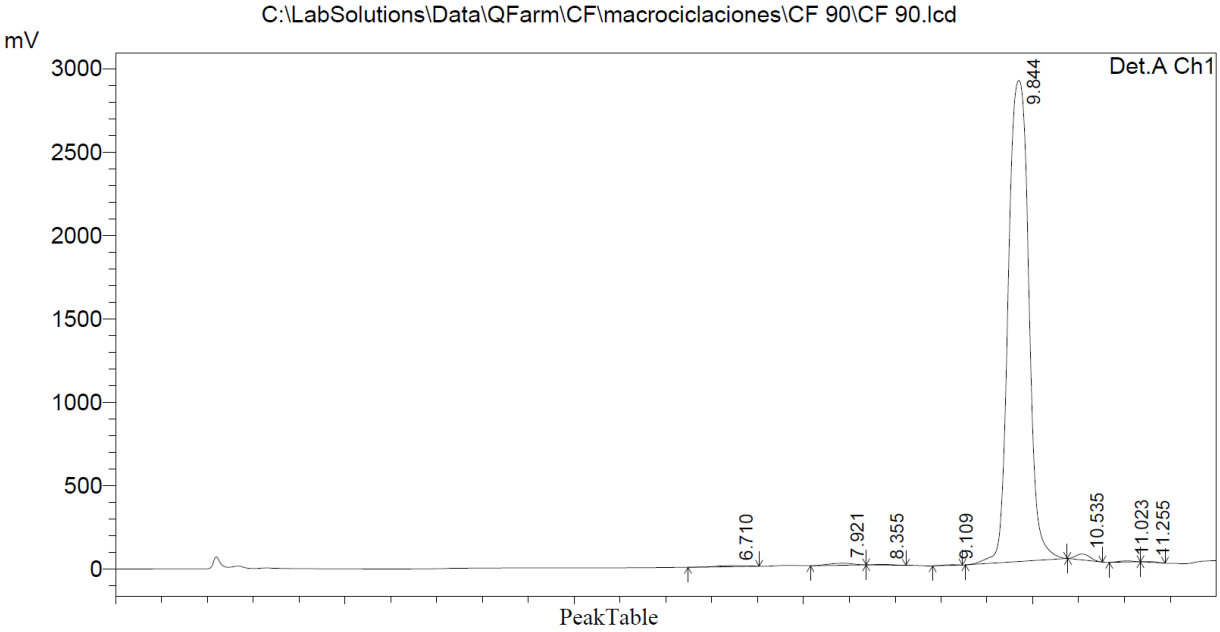


2

NMR in $CDCl_3$



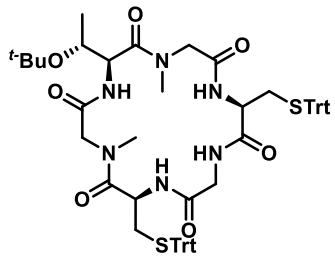

170.83
170.46
170.37
169.35
168.88
168.70
144.38
144.32
129.54
128.13
126.96
77.35
77.24
77.03
76.71
75.07
67.70
67.36
54.56
54.14
53.28
52.20
43.13
43.00
39.21
32.74
31.77
28.17
18.50
2E+07
2E+07
2E+07
2E+07
2E+07
1E+07
1E+07
1E+07
8E+06
6E+06
4E+06
2E+06
0
-2E+06
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)


C:\LabSolutions\Data\QFarm\CF\macrociclaciones\CF 90\CF 90.lcd
mV
3000
2500
2000
1500
1000
500
0
Det.A Ch1
9.844
6.710
7.921
8.355
9.109
10.535
11.023
11.255
PeakTable

Detector A Ch1 220nm

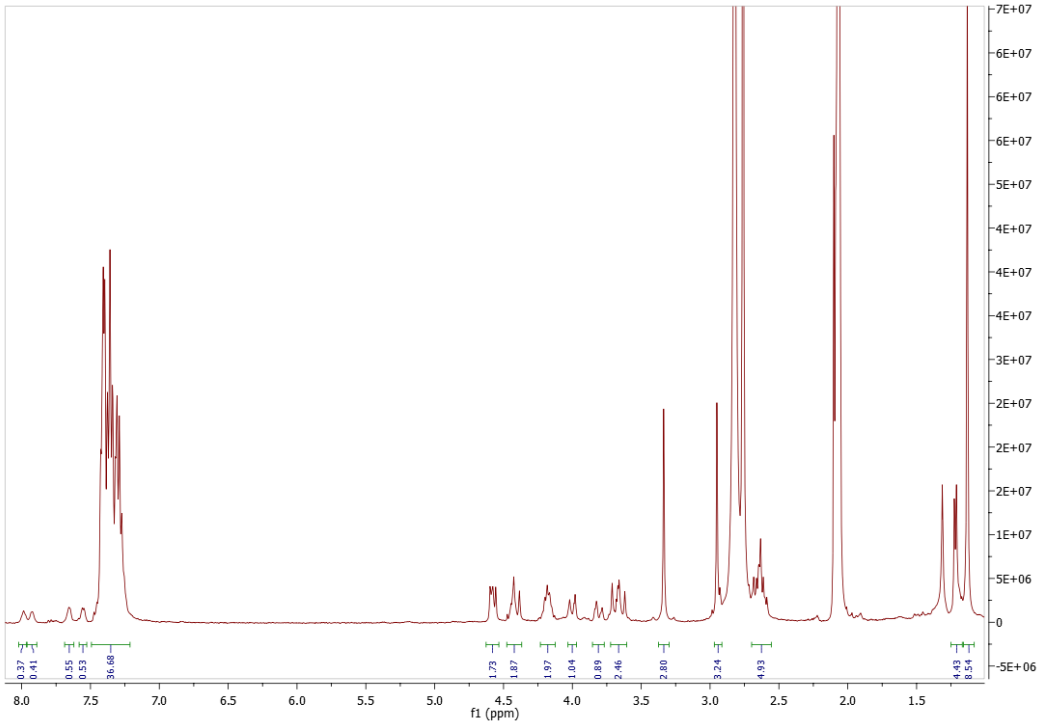
Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.710	135023	6965	0.296	0.236
2	7.921	222459	12156	0.488	0.411
3	8.355	43168	2902	0.095	0.098
4	9.109	34924	3503	0.077	0.119
5	9.844	44650360	2884452	97.992	97.604
6	10.535	373159	35085	0.819	1.187
7	11.023	71611	6703	0.157	0.227
8	11.255	34786	3497	0.076	0.118
Total		45565490	2955264	100.000	100.000

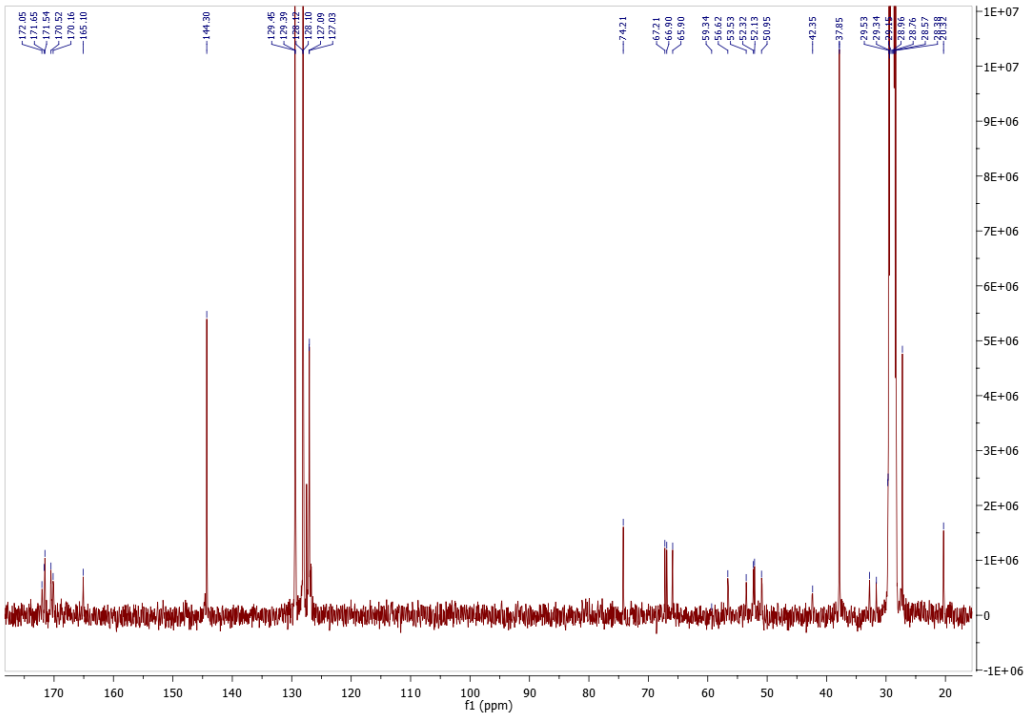
Cyclo-[NMe-Gly-_L_-Thr(^{t}Bu)-NMe-Gly-_L_-Cys(Trt)-Gly-_L_-Cys(Trt)] (3)

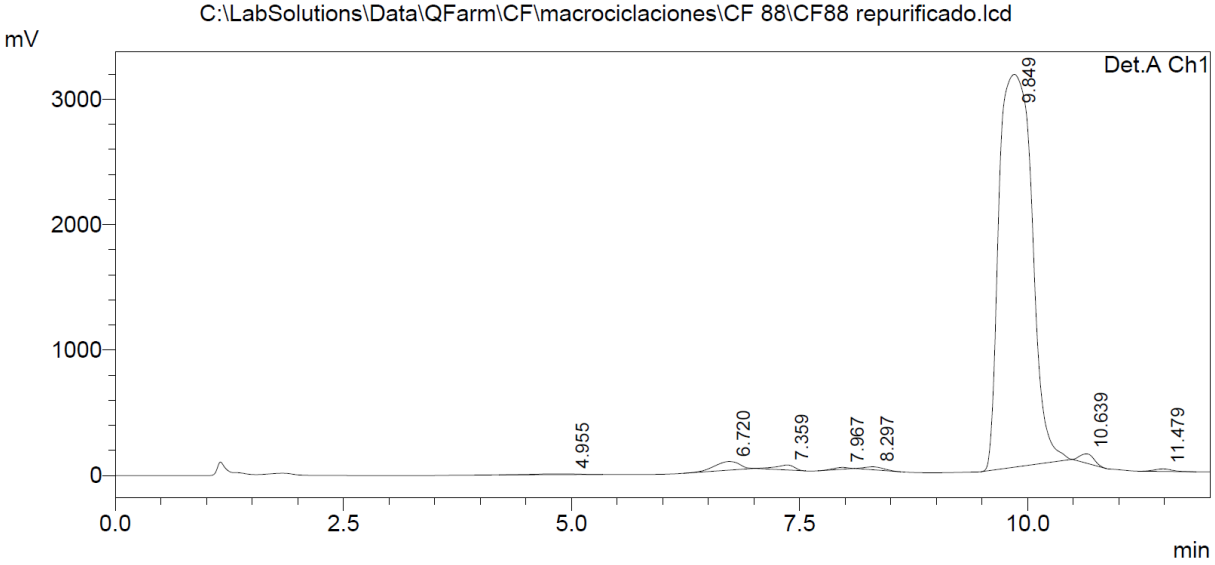


3

NMR in $(CD_3)_2CO$

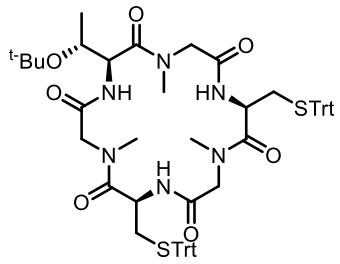






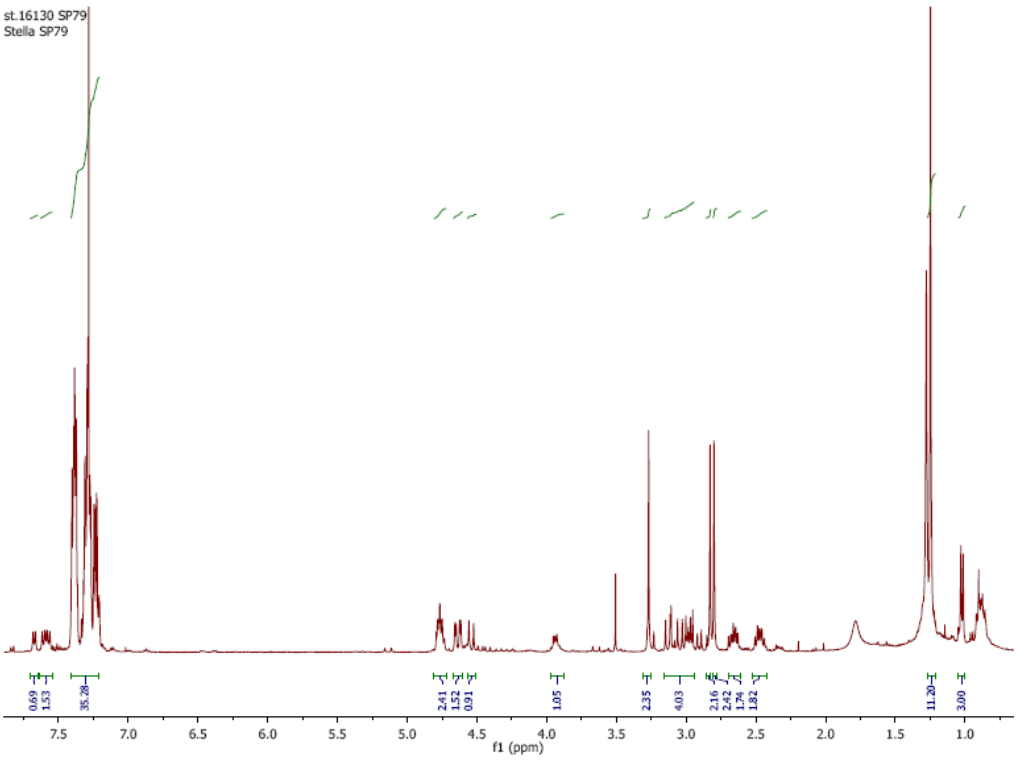
Peak#	Name	Ret. Time	Area	Height	Area %	Height %
1		4.955	230883	6521	0.280	0.193
2		6.720	1317911	68868	1.600	2.039
3		7.359	561537	39031	0.682	1.156
4		7.967	171566	14710	0.208	0.436
5		8.297	339073	22753	0.412	0.674
6		9.849	78699210	3131136	95.533	92.726
7		10.639	789960	73721	0.959	2.183
8		11.479	268974	20024	0.327	0.593
Total			82379115	3376763	100.000	100.000

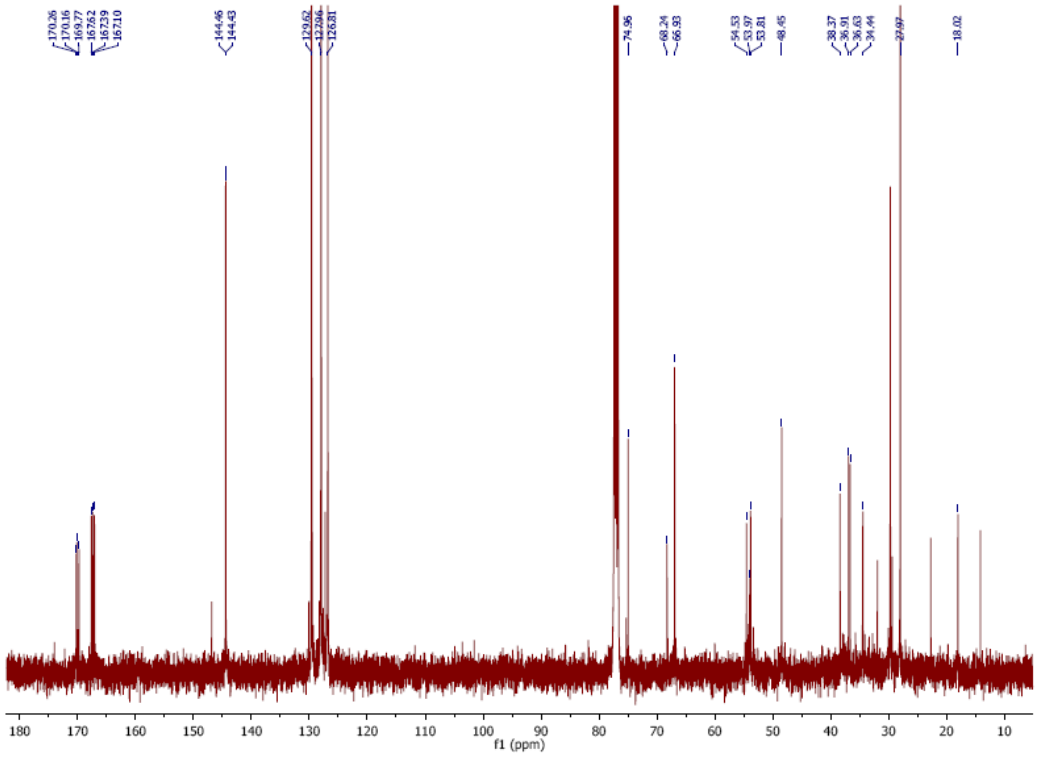
Cyclo-[L-Thr(ᵗBu)-NMeGly-L-Cys(Trt)-NMeGly-L-Cys(Trt)-NMeGly] (4)

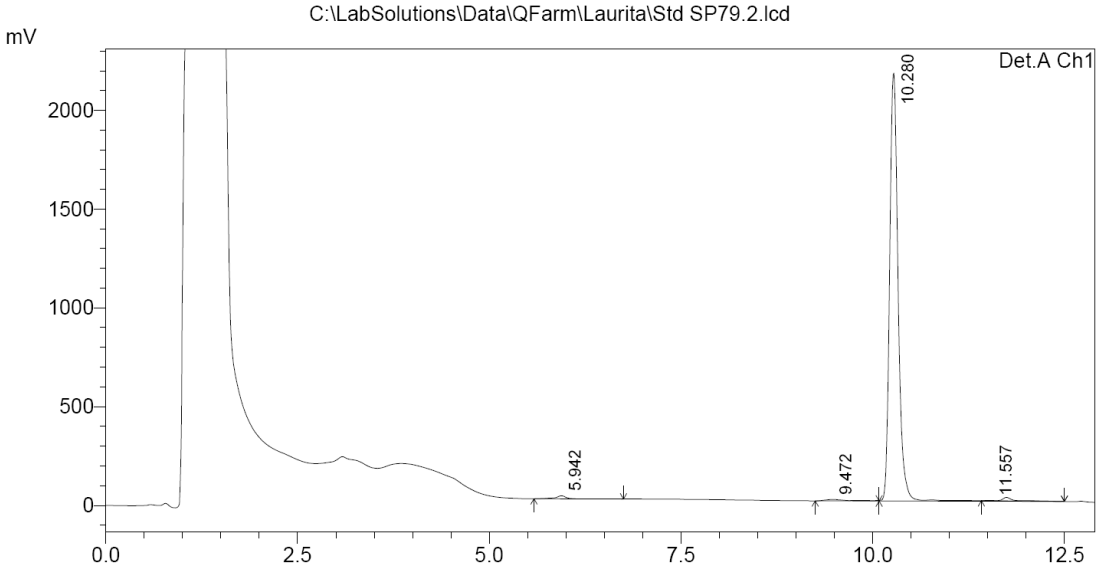


4

NMR in $CDCl_3$





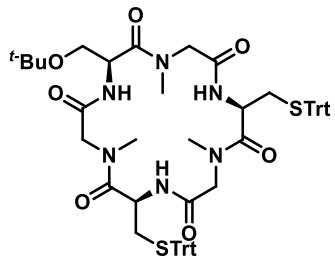


PeakTable

Detector A Ch1 220nm

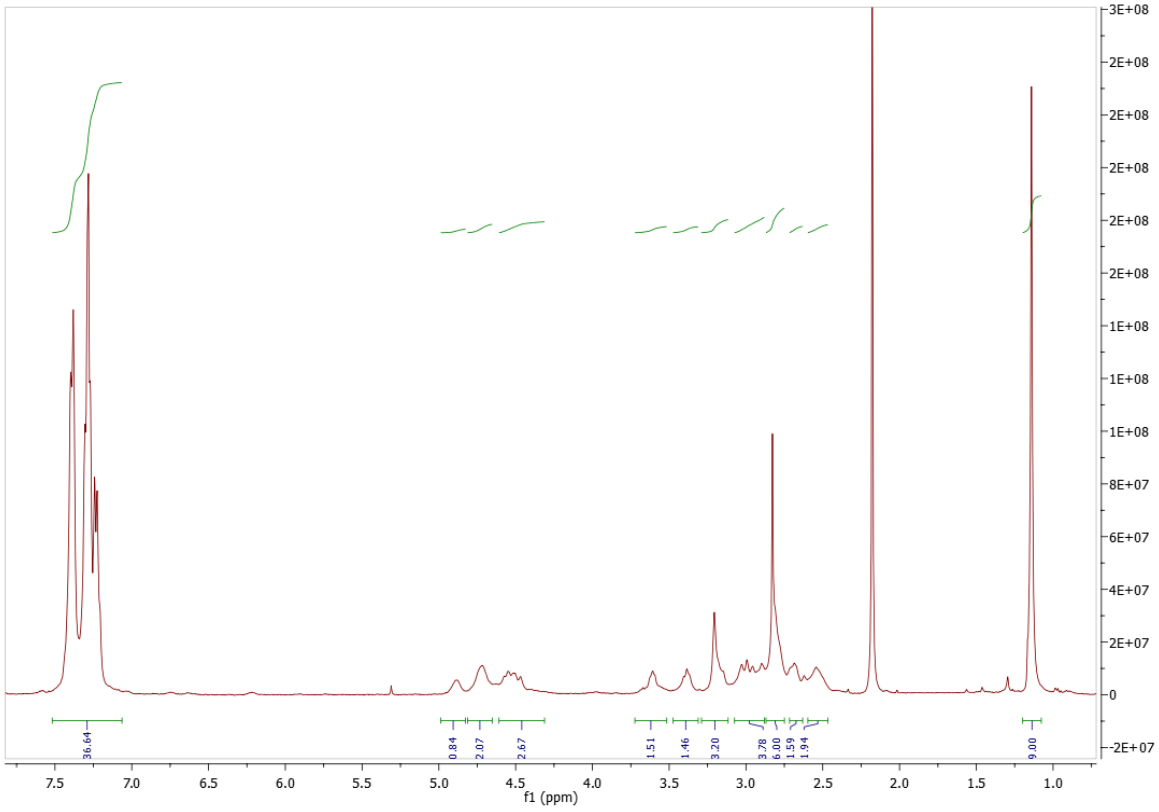
Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.942	152567	13098	0.885	0.728
2	9.472	162085	6656	0.940	0.370
3	10.280	16740203	1777790	97.119	98.811
4	11.557	181897	1633	1.055	0.091
Total		17236753	1799178	100.000	100.000

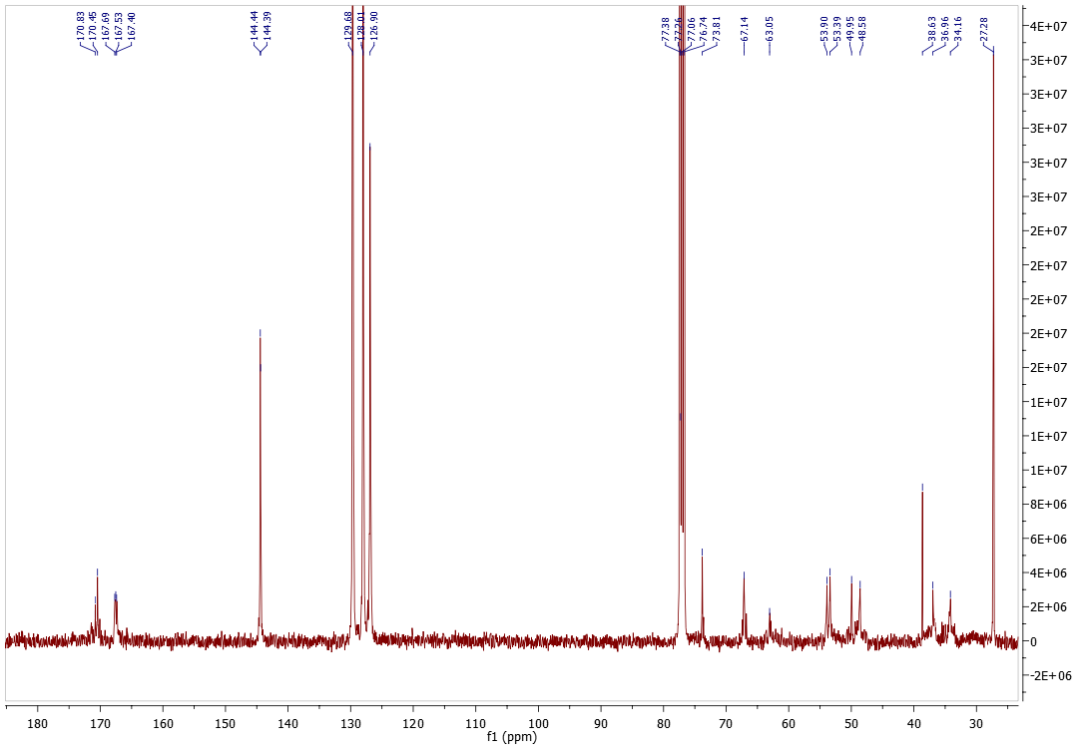
***Cyclo*-[NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Ser(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)] (5)**

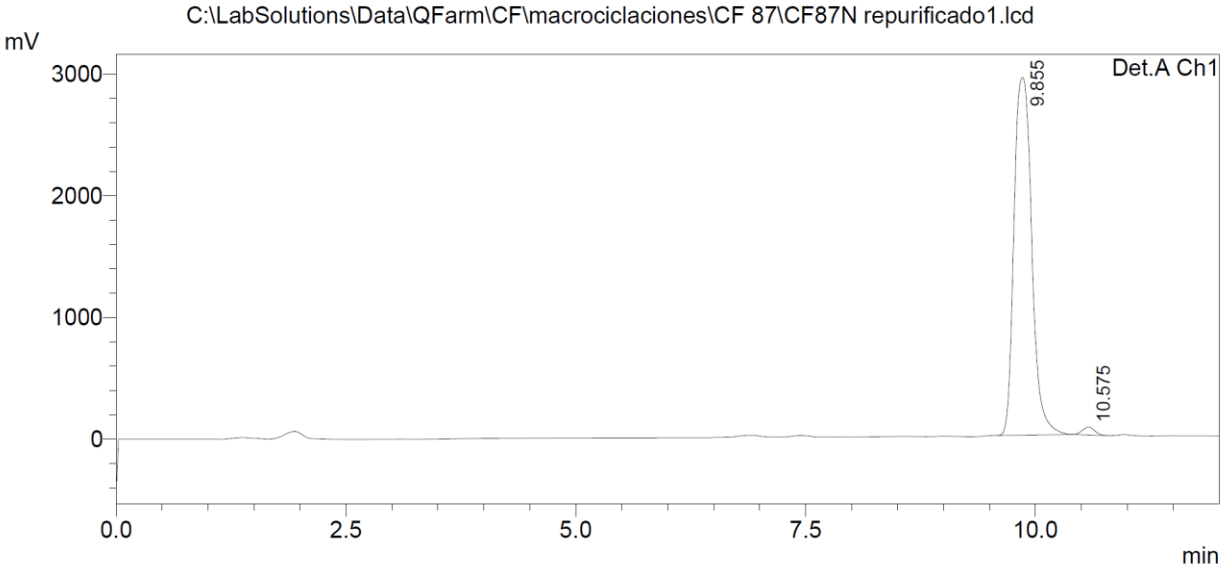


5

NMR in $(CD_3)_2CO$

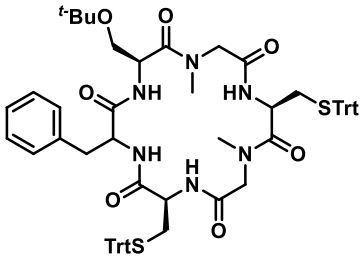






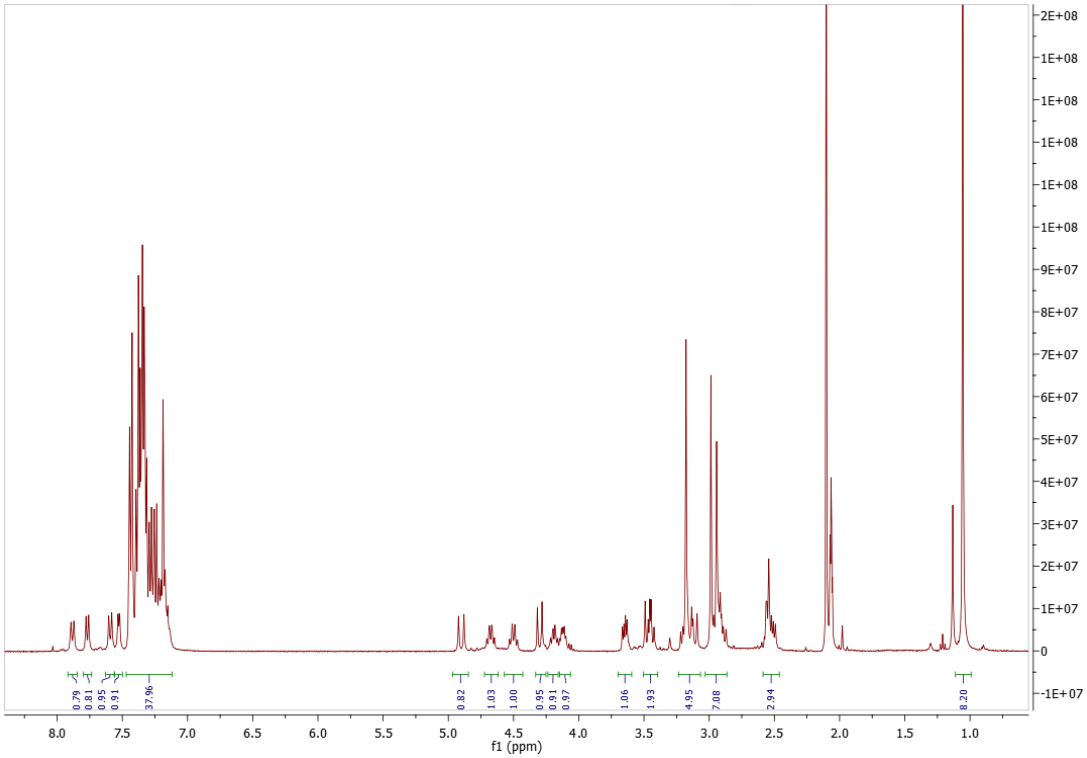
Peak#	Name	Ret. Time	Area	Height	Area %	Height %
1		9.855	39537943	2941053	98.502	97.840
2		10.575	601421	64921	1.498	2.160
Total			40139365	3005974	100.000	100.000

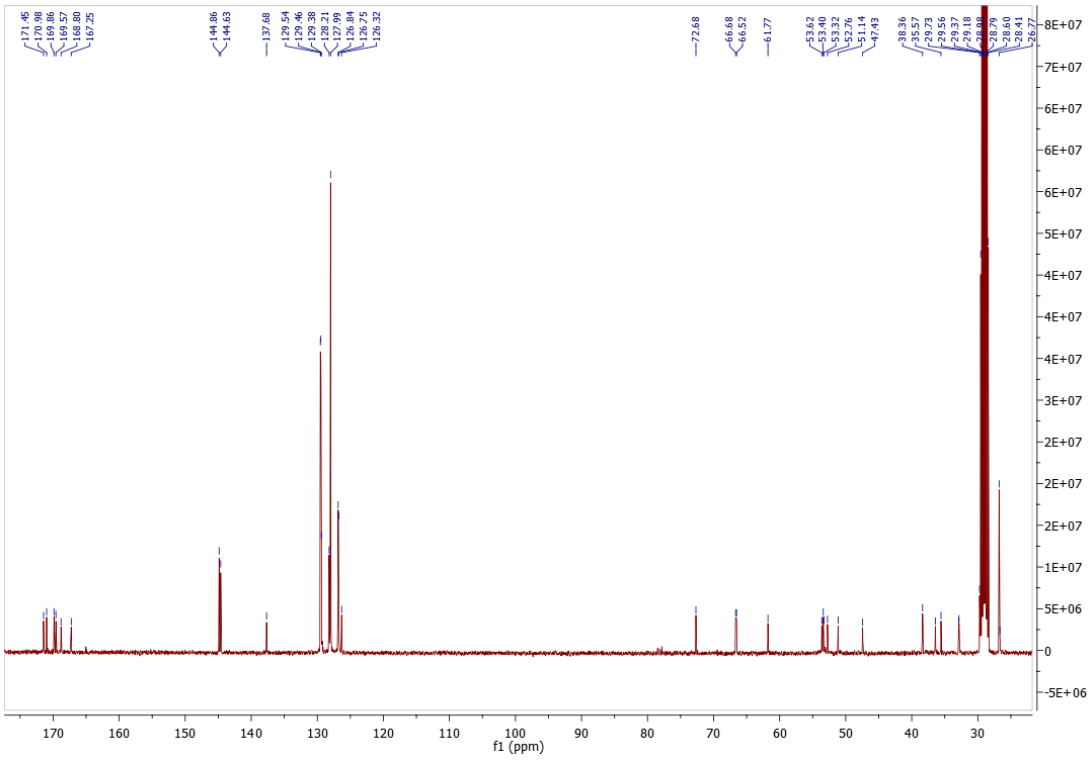
Cyclo-[NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Cys(Trt)-*L*-Phe-*L*-Ser(^{t}Bu)] (6)

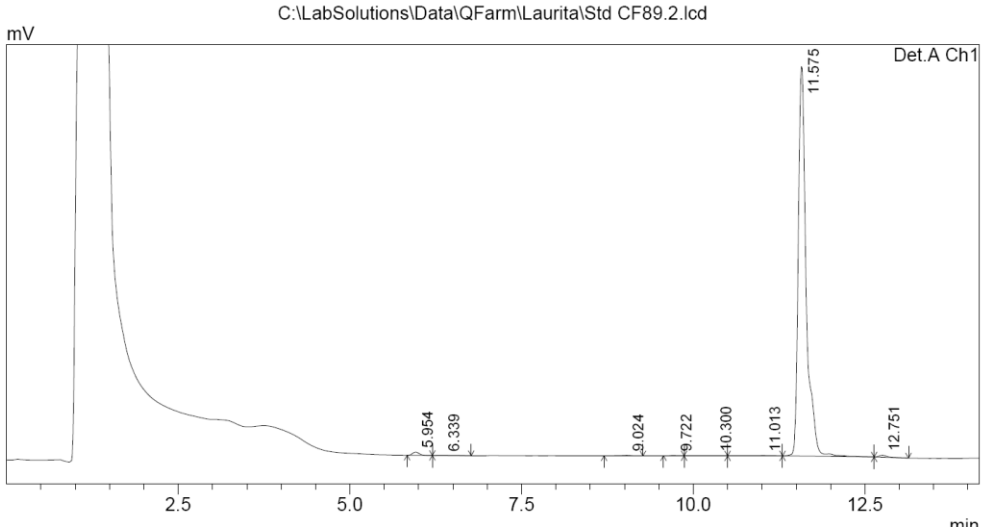


6

NMR in $(CD_3)_2CO$





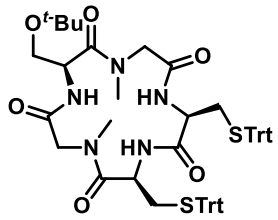


PeakTable

Detector A Ch1 220nm

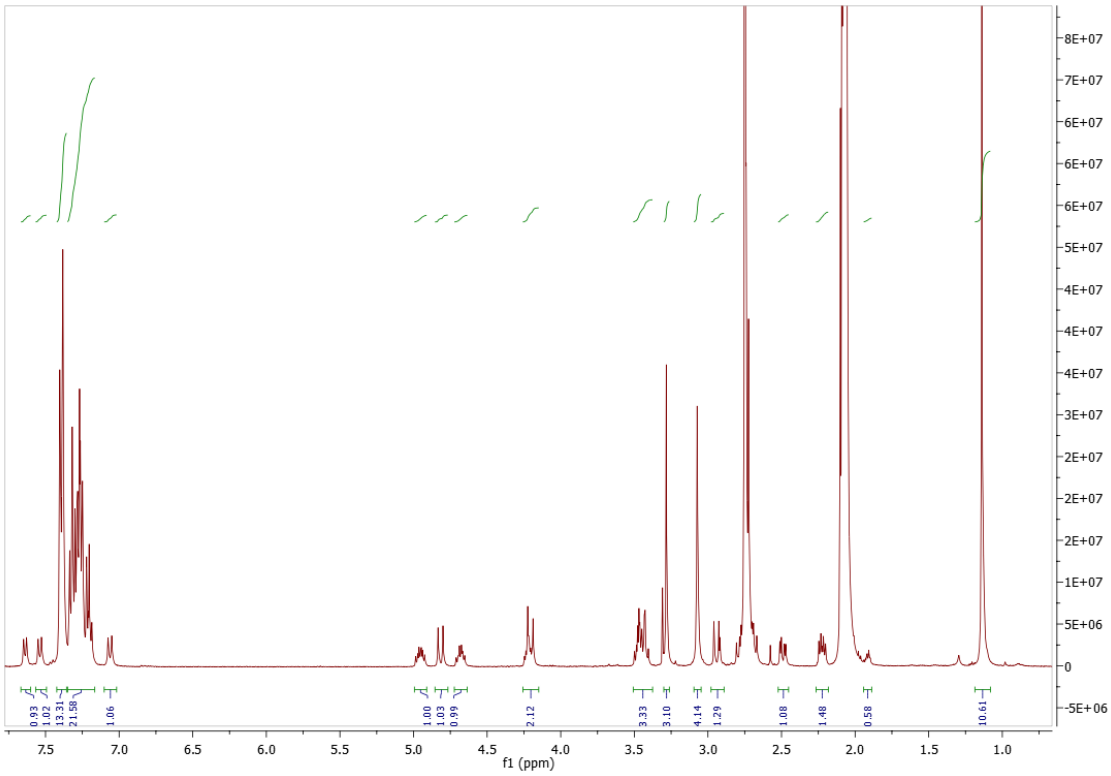
Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.954	125474	18455	0.673	0.826
2	6.339	17218	1055	0.092	0.047
3	9.024	38080	2903	0.204	0.130
4	9.722	17815	2234	0.095	0.100
5	10.300	19517	812	0.105	0.036
6	11.013	44902	1754	0.241	0.078
7	11.575	18305837	2197086	98.126	98.281
8	12.751	86564	11216	0.464	0.502
Total		18655407	2235516	100.000	100.000

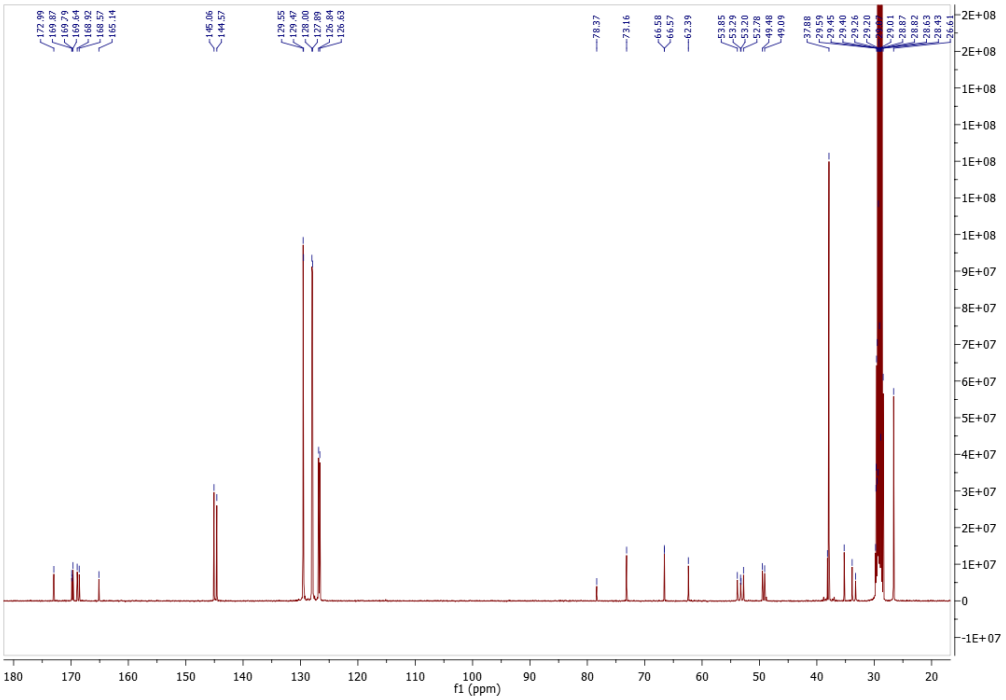
***Cyclo*-[NMe-Gly-*L*-Ser(^{t}Bu)-NMe-Gly-*L*-Cys(Trt)-*L*-Cys(Trt)] (7)**

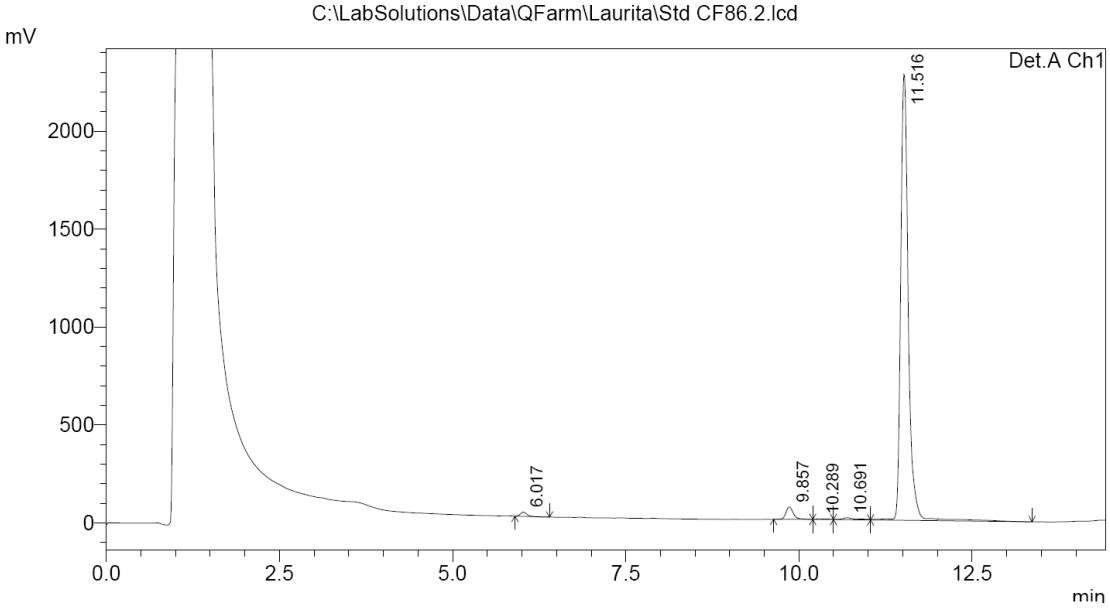


7

NMR in $(CD_3)_2CO$





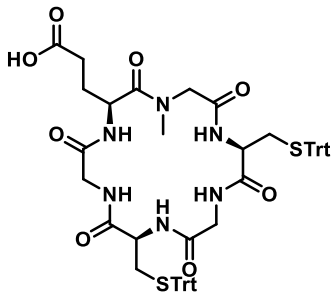


PeakTable

Detector A Ch1 220nm

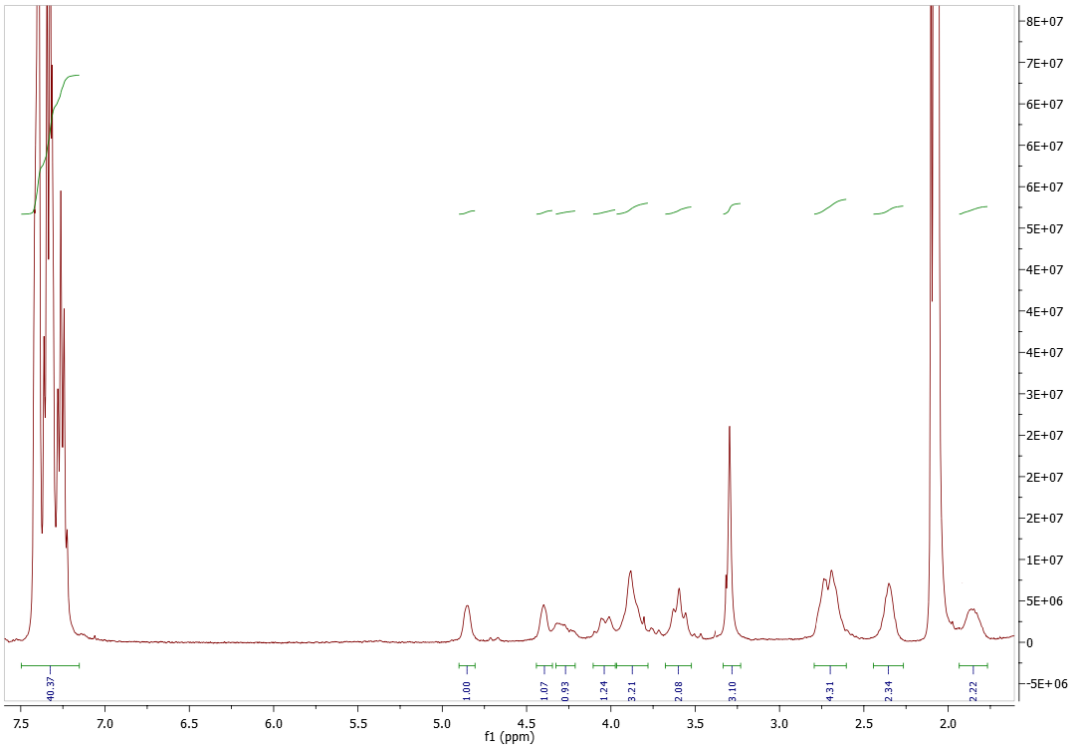
Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.017	135940	20834	0.741	0.894
2	9.857	458703	60688	2.499	2.604
3	10.289	28482	2197	0.155	0.094
4	10.691	152757	9964	0.832	0.428
5	11.516	17577469	2236779	95.773	95.980
Total		18353352	2330462	100.000	100.000

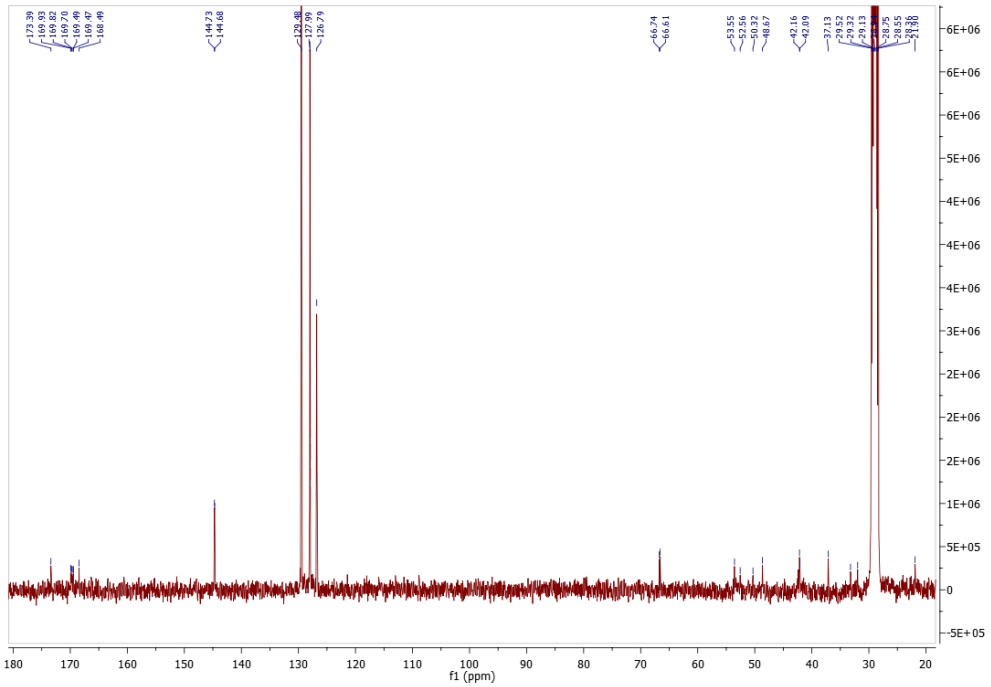
Cyclo-NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-Gly-*L*-Glu (8)

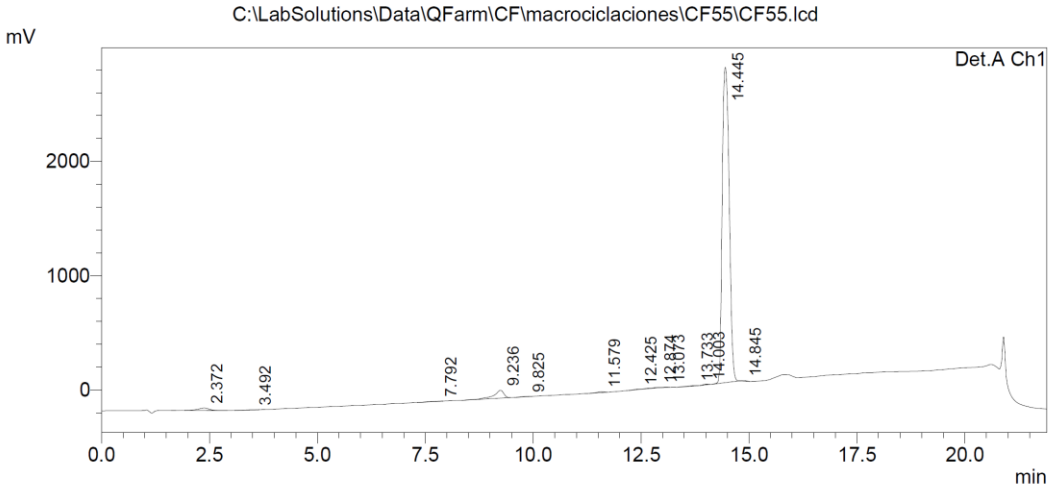


8

NMR in $(CD_3)_2CO$

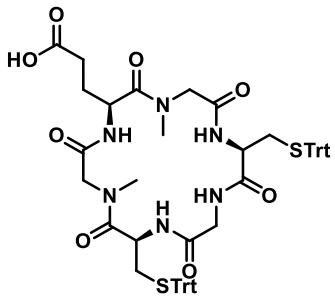






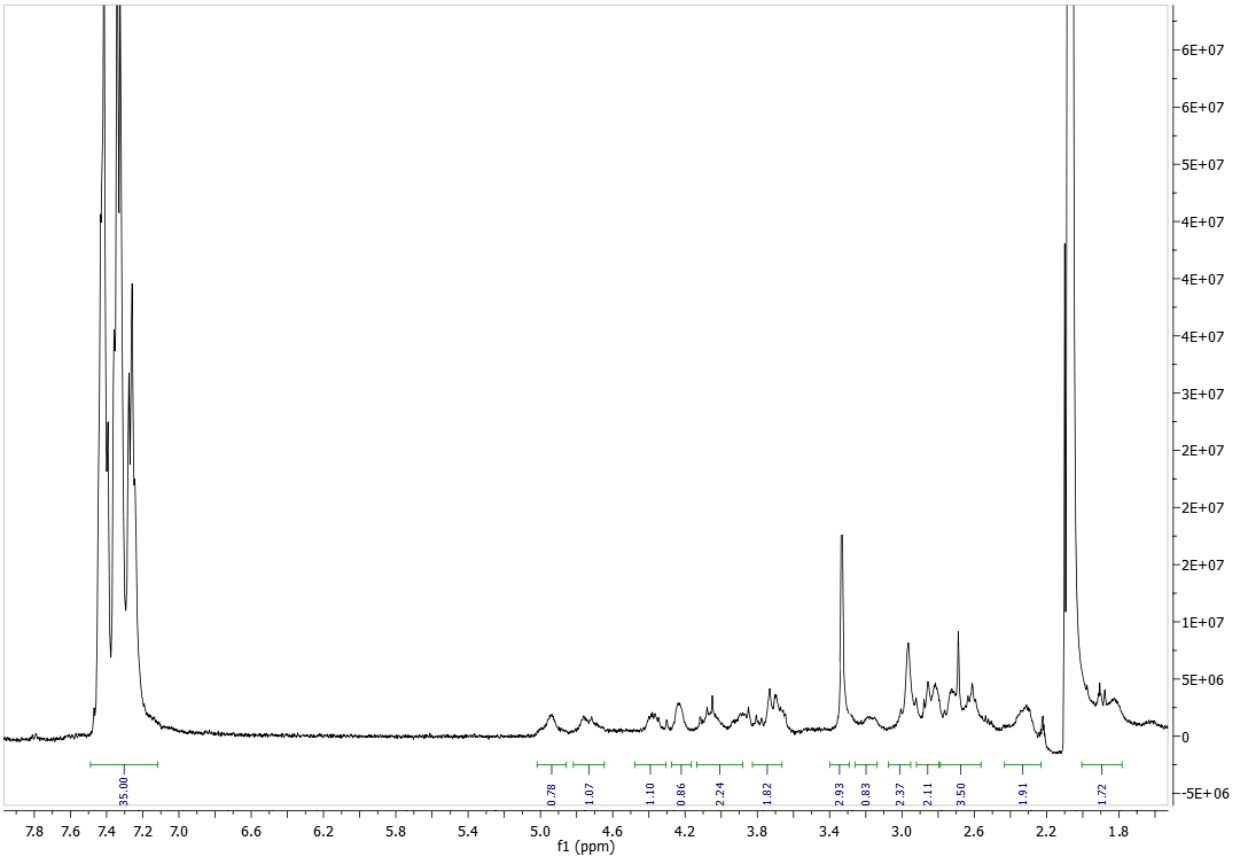
Peak#	Name	Ret. Time	Area	Height	Area %	Height %
1		2.372	374176	21881	1.143	0.758
2		3.492	18789	1129	0.057	0.039
3		7.792	51560	3318	0.158	0.115
4		9.236	1101193	68774	3.365	2.384
5		9.825	24340	2050	0.074	0.071
6		11.579	77782	6740	0.238	0.234
7		12.425	25680	2643	0.078	0.092
8		12.874	64595	3958	0.197	0.137
9		13.073	15003	2600	0.046	0.090
10		13.733	51167	3146	0.156	0.109
11		14.003	43943	5754	0.134	0.199
12		14.445	30841984	2758637	94.237	95.609
13		14.845	37968	4711	0.116	0.163
Total			32728179	2885342	100.000	100.000

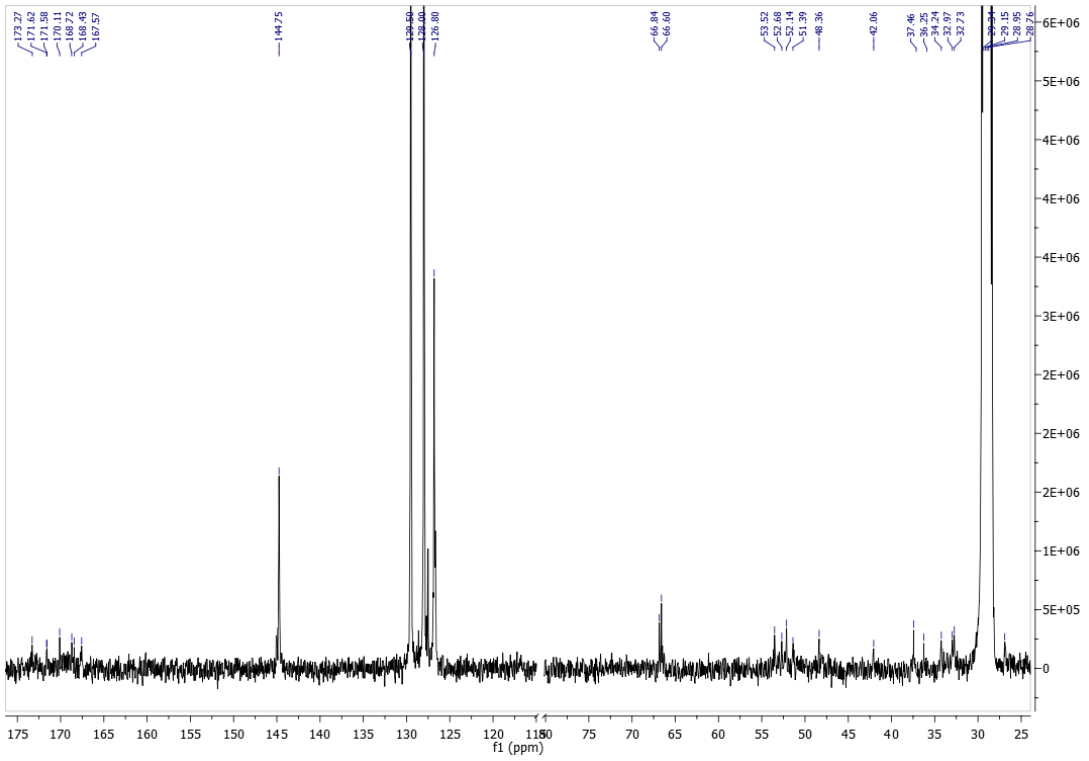
Cyclo-[NMe-Gly-*L*-Cys(Trt)-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Glu] (9)

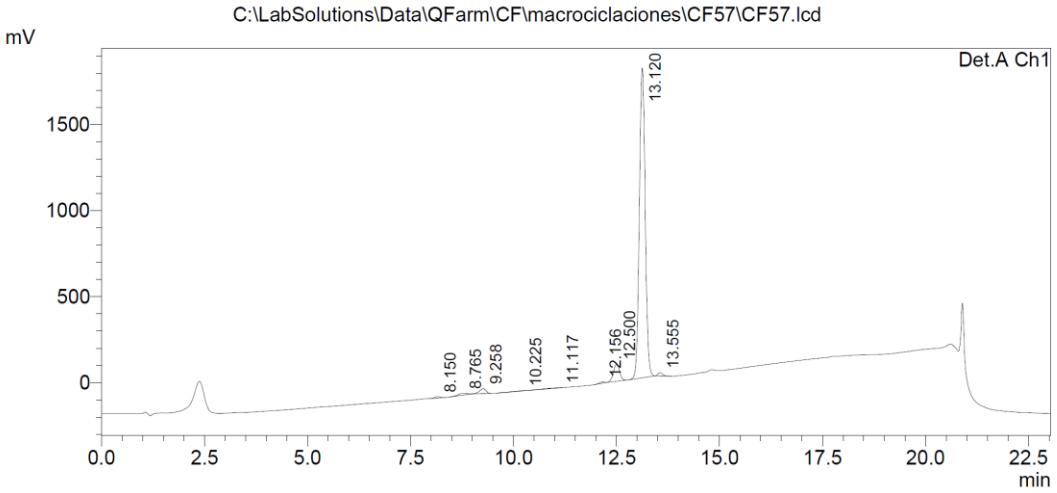


9

NMR in $(CD_3)_2CO$

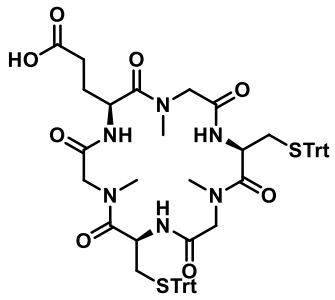






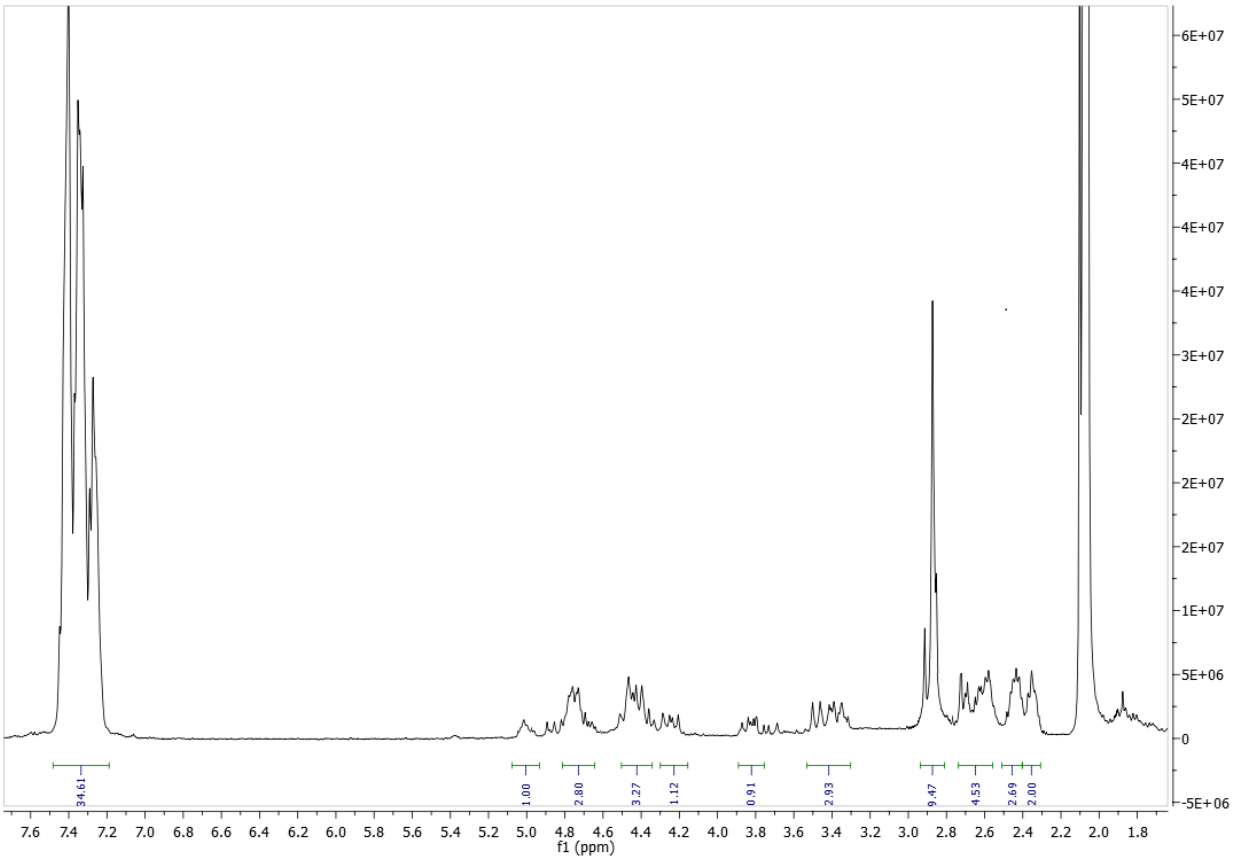
Peak#	Name	Ret. Time	Area	Height	Area %	Height %
1		8.150	77281	7943	0.391	0.401
2		8.765	165588	10911	0.839	0.551
3		9.258	324345	29710	1.643	1.500
4		10.225	49240	1227	0.249	0.062
5		11.117	78738	1086	0.399	0.055
6		12.156	65674	7619	0.333	0.385
7		12.500	1049088	100907	5.314	5.093
8		13.120	17774809	1802836	90.030	90.993
9		13.555	158393	19061	0.802	0.962
Total			19743154	1981300	100.000	100.000

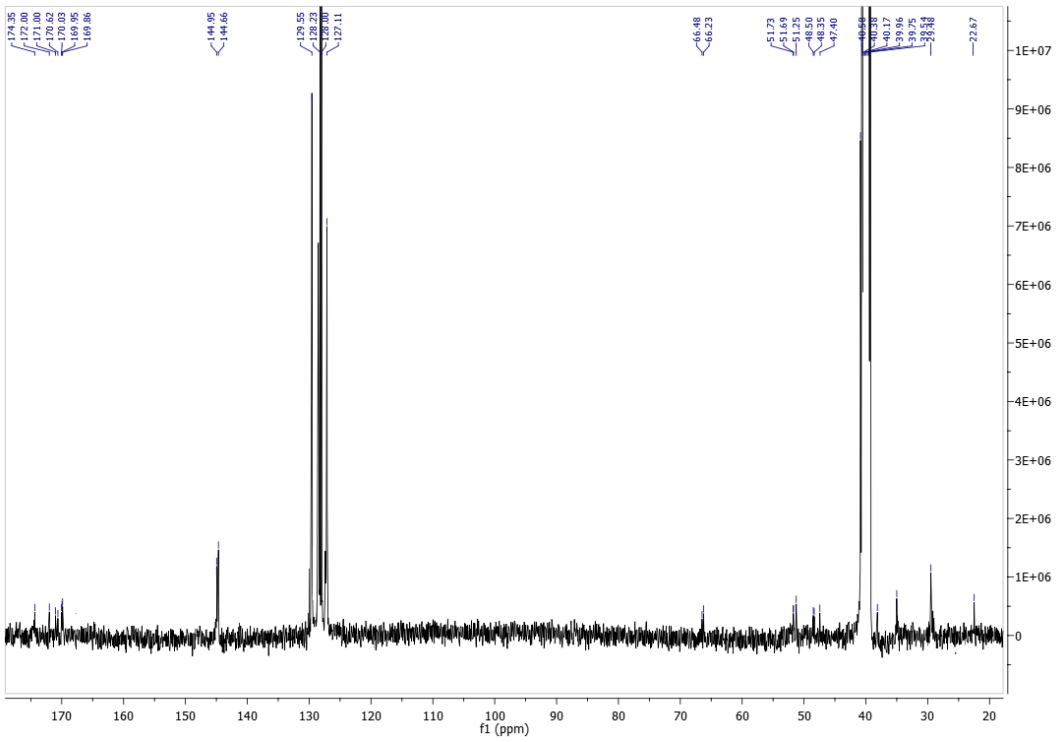
Cyclo-[NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Cys(Trt)-NMe-Gly-*L*-Glu] (10)

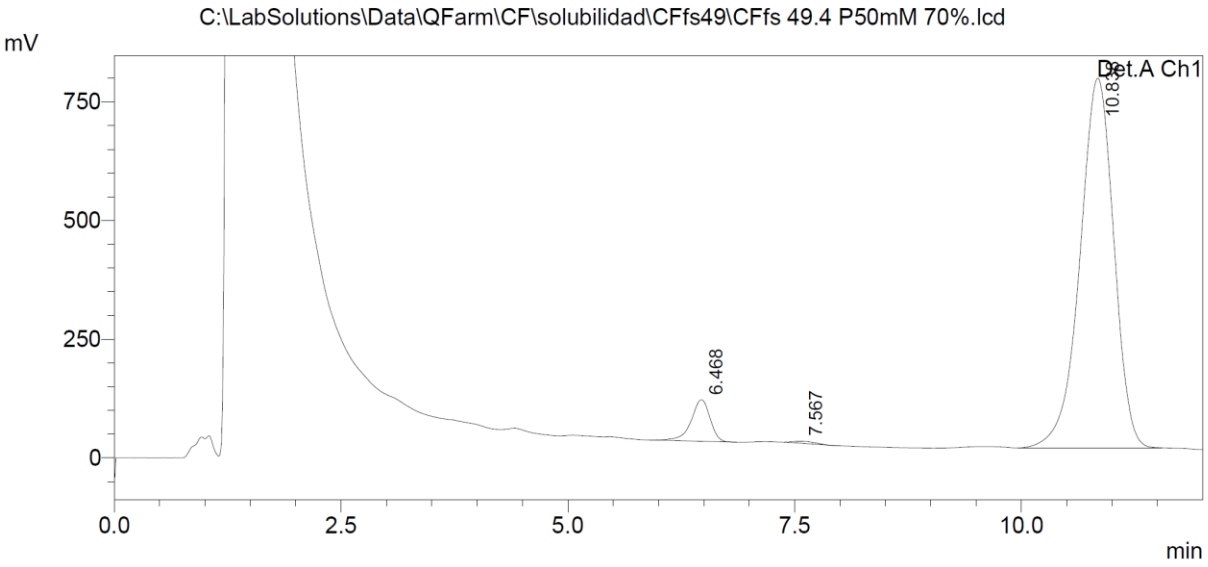


10

NMR in $(CD_3)_2CO$







Peak#	Name	Ret. Time	Area	Height	Area %	Height %
1		6.468	1272875	87673	5.896	10.064
2		7.567	71302	4617	0.330	0.530
3		10.838	20244730	778883	93.774	89.406
Total			21588908	871173	100.000	100.000

6. Physicochemical properties determination

All the solvents used for the tests and for the chromatography, were HPLC quality.

The determinations of solubility, stability and Log P were made in a Shimadzu analytical HPLC (pump LC-10AT) equipped with a SPD20A Prominence UV/Vis detector. The determination of solubility and stability, were performed using a Phenomenex LunaR SV 100A C18 column (150 x 4.6 mm, 5 μm). The determination of Log P was performed using an Agilent Zorbax Eclipse XDB-C8 column (250 x 4.6 mm, 5 μm). The determination of concentrations for PAMPA assay was performed in an Agilent Infinity 1260 analytical HPLC analysis equipped with a UV/Vis DAD detector, with a Phenomenex LunaR SV 100A C18 column (150 x 4.6 mm, 5 μm).

6.1 Determination of Solubility and Stability.

Solubility and Stability at pH = 7.4: 5 μL of a 10 mM solution, of the compound to be analyzed, in DMSO was taken and diluting up to 100μL with phosphate buffered saline solution pH = 7.4 (0.1M). This solution was allowed to equilibrate at 37 ° C, with stirring (100 rpm) for 2 h.

The resulting solution was filtered and its concentration was determined by HPLC with UV detector at λ = 220 nm, using an external standard of the product.

Solubility and Stability at pH = 1.0: 5 μL of a 10 mM solution, of the compound to be analyzed, in DMSO was taken and diluting up to 100 μL with hydrochloric acid solution pH = 1.0 (0.1 M). This solution was allowed to equilibrate at 37 ° C, with stirring (100 rpm) for 2 h.

The resulting solution was filtered and its concentration was determined by HPLC with UV detector at λ = 220 nm, using an external standard of the product.

The areas were determined for the peaks of the test solution and for the standard peak for each test product, running each sample in a H_2O/MeCN, 0.003M TFA system: (the composition of the mobile phase depends of the compound to be analyzed) on a Phenomenex column C18 Luna SV 100A, 5 μm, 4.6 x 150 mm, with a flow of 1.5 mL / min, measuring the absorbance at 220 and 280 nm.

$$[\text{Product}] = \frac{A_P \times [\text{Standard}]}{A_E}$$

[Product] = Product concentration
[Standard] = Concentration of the Standard
A_P = Peak area for product.
A_E = Peak area for standard

6.2 Determination of Log P.

The determination of Log P was made based on the OECD Guidelines for the Testing of Chemicals (117): Partition coefficient (n-octanol / water) Hight Performance Liquid Chromatography (HPLC) Method, for determination of the partition coefficient by HPLC.

The retention times (t_R) of the different test compounds were determined, running each sample in an isocratic system H_2O: MeCN (25:75), on a Zorbax XDB-C8, 5µm, 4.6 x 25 mm column, with a flow of 1mL/min, the absorbance was determined at 220 and 254 nm, in order to interpolate this value in the linear regression equation obtained with the values of the standards.

Standards: Uracil (t_0), Nitrobenzene (Log P = 1.9), Bromobenzene (Log P = 3.0), Naphthalene (Log P = 3.6), Benzylbenzoate (Log P = 4.0), and Testosterone Enanthate (Log P = 8.3).

STANDARS	t_R	k	log k	log P
Uracil	2.4755	0		
Nitrobenzene	3.8545	0.557	-0.2641	1.9
Bromobenzene	5.3755	1.1714	0.0687	3
Naphthalene	5.6575	1.2854	0.1090	3.6
Benzylbenzoate	5.9245	1.3933	0.1440	4.0
Testosterone Enanthate	27.3805	10.0606	1.0026	8.3

$$k=\frac{t_R - t_0}{t_0}$$

$$\text{Log P} = 3.0482 + 5.2011 \text{ Log k}$$

k= Capacity factor..
t_R= Retention time
t_0= Dead Time (t_R of Uracil)
Log k= Log of the capacity factor
Log P= Log of the partition coefficient

6.3 PAMPA trans-membrane permeability determination.

The assay was performed following the general PAMPA protocol of Lokey Lab Protocols.

Solutions 20 µM / 5% DMSO in PBS buffer: 50 µL of a 400 µM solution of each compound was taken and 950 µL of PBS buffer was added.

Donor Plate (Plate D): On the wells to be used of this plate, 5 µL of a 1% Lecithin solution was added in Dodecano, being careful not to touch the bottom membrane of this plate. This was allowed to moisten for 10 minutes. After 10 minutes, plate D is loaded with 180 µL of the 20 µM solutions of each compound in triplicate, including a blank of a 5% DMSO solution in PBS and the standard solutions.

Acceptor plate (plate A): On the wells to be used of this plate, 180 µL of a solution of 5% DMSO in PBS (pH = 7.4, 1 M) was added using a multichannel pipette. Once complete it is covered to avoid evaporation.

Then plate D was placed on Plate A, taking care that no bubbles remain between both plates, and covered to avoid evaporation. They were placed in a shaker with shaking at 100 rpm for 20 hours.

After that time, both plates were separated, and the one that was not in use was covered. The solution from each well was taken with a syringe and filtered by passing it to a vial and then to determine the concentration of the analyte therein by HPLC. For plate D, care must be taken not to touch the membrane with the tip of the syringe.

$$Pe = \left(-C \times \ln\left(1 - \frac{[analyte]_a}{[analyte]_{eq}}\right)\right)$$

$[analyte]_a$: concentration of the analyte in the acceptor

$[analyte]_{eq}$: analyte concentration in equilibrium (analyte dissolved, not adhered or retained in membrane or precipitated in the solution)

$$C = \frac{V_d \times V_a}{(V_d + V_a) \times M_{sa} \times T_s} = \frac{(180 \times 180)\ \mu L^2}{(180 + 180)\ \mu L \times 240\ mm^2 \times 72000\ s} = 5.24 \times 10^{-6}\ \mu L/mm^2.\ s$$

V_d= Donor Volume = 180 µL

V_a= Acceptor Volume= 180 µL

T_s= Test time in seconds = 72000 s

M_{sa}= Active surface of the membrane = 240 mm^2

C= Test constant.

$$[analyte]_{eq} = \frac{m\ anal_{acceptor} + m\ anal_{donor}}{V_a + V_d}$$

$$m\ anal_{acceptor} = [analyte]_a \times V_a$$

$$m\ anal_{donor} = [analyte]_d \times V_d$$

$[analyte]_a$: is determined by the I_a

I_a= Acceptor Intensity

$[analyte]_d$: is determined by the I_d

I_d= Donor Intensity

$$[analyte]_{eq} = \frac{I_a \times V_a + I_d \times V_d}{V_d + V_a} = \frac{180\ (I_a + I_d)}{360} = \frac{I_a + I_d}{2}$$

substituting in the equation of Pe

$$Pe = \left(-C \times \ln\left(1 - \frac{2 \times I_a}{I_a + I_d}\right)\right)$$

When I_a has a value similar to I_d the data is discarded

7. Biological Evaluation Procedures

7.1 Procedure for in vitro evaluation against *Plasmodium falciparum*.

P. falciparum K1

Parasite crops: K1 strain of *P. falciparum* from Thailand that is resistant to chloroquine and pyrimethamine but sensitive to mefloquine was used. The cultures are naturally asynchronous and are maintained in a continuous logarithmic growth phase in RPMI 1640 medium supplemented with 5% washed human A + erythrocytes, 25 mM HEPES, 32 nM $NaHCO_3$ and AlbuMAXII (lipid rich bovine serum albumin) (GIBCO, Grand Island, NY) (CM). All cultures and tests are carried out at 37°C under an atmosphere of 5% CO_2 and 5% O_2, with a balance of N_2.

Sensitivity tests to the compounds: the stock solutions are prepared in 100% DMSO (dimethylsulfoxide) at 20 mM. The compound is further diluted to the appropriate concentration using complete RPMI1640 medium supplemented with 15 nM cold hypoxanthine and AlbuMAXII. The assays are performed in sterile 96-well microtiter plates, each plate contains 100 µl of parasite culture (0.5% parasitaemia, 2.5% hematocrit). Each product is analyzed in triplicate and the growth of the parasite is compared with the control and the wells of the target (uninfected erythrocytes). After 24 h of incubation at 37°C, 3.7 Bq of [3H] hypoxanthine is added to each well. The cultures are incubated for another 24 h before they are harvested on fiberglass filter mats. Radioactivity is counted using a Wallac Microbeta 1450 scintillation counter. The results are recorded as counts per minute (CPM) per well at each concentration of product, control and blank wells. The percent inhibition is calculated from the comparison with the blank and control wells, and the EC_{50} values are calculated using Prism ™.

Test: the compound is diluted three times to 12 different concentrations with an appropriate starting concentration. The EC_{50} is determined by a sigmoidal dose response using Prism ™. For each assay, the EC_{50} value for the parasite against the known antimalarial chloroquine and artesunate, plus other standard compounds appropriate for the assay, is determined.

P. falciparum 3D7

The tested compounds and the control drug (artesunate) were used to prepare two stock solutions in DMSO: solution A (dilution of powder sample) at 20 mM, and solution B at 2 mM (made by dilution of solution A). Next, the compounds were diluted in RPMI culture medium according to the desired test concentration. The 10 µM test solution was the most concentrated among the

compounds tested. Thus, the maximum concentration of DMSO used in this assay was 0.5%. No type of precipitation of the diluted compounds was observed for the assay.

Test conditions: 2% hematocrit, 0.5% initial parasitaemia, with artesunate and pyrimethamine as control compounds for experiment validation. The EC50 value of each compound was evaluated in at least two independent experiments. The infected and uninfected erythrocytes were used as negative and positive controls, respectively. After 72 hours of incubation the SYBR Green assay was performed as described by Smilkstein et al. (Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J.X.; Wilairat, P.; Riscoe, M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob. Agents Chemother. 2004, 48, 1803)

7.2 Procedure for in vitro cytotoxicity against HepG2 cells and murine macrophages

Cytotoxicity assay against HepG2 cells

The tetrazolium MTT dye assay was used to evaluate the cytotoxicity of the active compounds. Briefly, HepG2 cells were distributed in a 96-well plate at a concentration of 5x104 cells per well and incubated overnight for adhesion. The compounds were diluted 8x (from a stock solution at 20 mM) in a solution of RPMI supplemented with 10% of fetal bovine serum. Next, a serial dilution was performed and 20 μL were transferred for the cell plate and incubated for more 24 hours. The compounds were tested at a final concentration of 250 to 3.9 μM. The highest concentration of DMSO used was 1.25%. A control with the vehicle was performed and used as a positive control of cell growth. The cell viability was measured by MTT as described for Denizot et al. (Denizot F., Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods. 1986;89:271–277.)

Cytotoxicity study on murine macrophages:

Cell line: J 774.

Culture medium: DMEM glutamax + 10% FCS and antibiotics (Pen-Strep).

Compounds: From the parent solutions of the compounds, dissolved in DMSO at a concentration of 10 mM, dilutions were prepared in medium to obtain final concentrations of 100, 20, 2, 0.2 and 0.002 μM.

Experiment: Cells were resuspended in fresh medium and plated in duplicate in 96-well plate at a cell density of 1.2 x 10 4 cells per well. The compounds were added after 24 h at the final concentrations of the previous point. After 24 hours, the culture medium was removed and the

wells were washed twice. The plates were incubated at 37 ° C, 5% CO_2 in a humidified oven. The cell counts were carried out in duplicate at 24 hs, using WST-1 reagent. Untreated cells with compounds and DMSO aggregates at final concentrations of 1%, as well as controls without cells were included as controls. After 3 hours the absorbance at 450 and 630 nm was measured in EL800 plate reader (Biotek). The corrected absorbance is determined as $A^{ic}450 = A^{i}450 - A^{i}630nm - Ablank450nm$. The result is expressed as percentage of cell growth with respect to the control without compound as a function of the concentration of compound and the corresponding EC_{50} is calculated

7.3 In vivo assays against *P. berghei*

A parasitic growth suppression assay *of P. berghei*, strain NK65 (originally received from the New York University Medical School) that infected mice was used as previously described (Peters et al., 1965).

Briefly, adult exogamic Swiss mice (20 ± 2 g) were inoculated with 1×10^5 red blood cells infected with *P. berghei*, intraperitoneally. The infected mice were kept together for at least 2 h and then randomly divided into groups of 3 animals per cage, which were subsequently treated with 50 mg / kg of each compound diluted in 3% DMSO (v / v) in RPMI administered daily by oral probe for three consecutive days.

Control groups were used in parallel, one treated with CQ (20 mg/kg) and the other with the vehicle. Smears were prepared from the mouse tails on days 5, 7 and 9 after infection and then fixed with methanol, stained with Giemsa and examined microscopically. The parasitemia and the percent growth inhibition of the parasite calculated in relation to the untreated control group (considered 100% growth) were evaluated using the following equation: [(C - T)/C] × 100; where C is the parasitaemia in the control group and T is the parasitaemia in the treated group.

7.4 Procedure for *in vivo* evaluation of plasma pharmacokinetic properties for compound 3.

The study was conducted at Sai Life Sciences Limited, Pune, India.

This study was carried out with the approval of the Institutional Committee of Animal Ethics (AICE) in accordance with the requirements of the Committee for the Purpose of Control and Supervision

of Animal Experiments (CPCSEA), India. The study was not conducted according to GLP regulations and was not audited by QA; however, all appropriate documentation is kept in the study file. The study group verified the accuracy of the study phases, the data generated and the report.

Healthy male Albino male mice (8-12 weeks of age) weighing between 25 and 35 g of NIB, India were used. Three mice were housed in each cage. The temperature and humidity were maintained at 22 ± 3ºC and 30-70%, respectively, and the lighting was controlled to give a sequence of 12 hours of light and 12 hours of darkness. Temperature and humidity were recorded by a self-controlled data logger system. All animals were given a laboratory diet for rodents throughout the study period, except 2 hours before dosing and 2 hours after fasting dose (Envigo Research private Ltd, Hyderabad).

Precisely weighed 19.93 mg of **3** for the dosage and added 0.112 ml of DMSO and vortexed. 3.617 ml of RPMI medium was added and vortexed. The final formulation was subjected to agitation for 2 minutes to obtain a finely suspended formulation. Aliquots of 200 µL were then taken for analysis. It was found that the formulations were within the acceptance criteria (the internal acceptance criteria are ± 20% of the nominal value).

Nine Swiss albino male mice were used in this study as a group. A solution formulation of **3** was administered in 3% DMSO and RPMI medium orally at 50 mg/kg. The dosage volume administered was 10 mL/kg. Blood samples were collected from the set of three mice at each time point on a labeled microcentrifuge tube containing K_2EDTA solution as an anticoagulant at 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h. The plasma samples were separated by centrifugation of whole blood and stored below -70 ± 10 ° C until the bioassay. All samples were processed for protein precipitation analysis using acetonitrile and analyzed with an LC-MS / MS method suitable for the purpose (LLOQ = 5.16 ng / ml). The pharmacokinetic parameters were calculated using the non-compartmental analysis tool of Phoenix WinNonlin® (Version 7.0).

Mean plasma concentration-time data of 3 following a single oral administration to male Swiss Albino mice (Dose: 50 mg/kg)

Mean Plasma Concentration (ng/mL)								
Time (hr)								
0.25	**0.5**	**1**	**2**	**4**	**6**	**8**	**12**	**24**
0.00	**9.04**	**20.93**	**61.13**	**40.86**	**16.43**	**22.62**	**10.78**	**0.00**

LLOQ = 5.16 ng/mL; No peaks or values below LLOQ were considered as zero for data analysis